CYSTEINE METABOLISM AND PANCREATIC NEUROENDOCRINE TUMOURS (PNETs) CHEMORESISTANCE

RAKHI CHANDA ROY

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research

Dissertação para obtenção do grau de Mestre em Investigação Biomédica

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

A

AA - Antibiotic-Antimycotic

ABC - ATP-Binding Cassette transporters

Akt – Protein Kinase B - serine/threonine-protein kinase

ARE - Antioxidant-Responsive Element

APUD- amine precursor uptake and decarboxylation

ATRX- α-thalassemia/mental retardation syndrome x-linked

ASCT - alanine -serine-cysteine transport system

ATP - Adenosine Triphosphate

B

BCRP - Breast Cancer Resistant Proteins

BRAF - Murine Sarcoma Viral Oncogene Homolog B

BSA - Bovine Serum Albumin

BSO - Buthionine Sulfoximine

C

CAFS - Cancer Associated-Fibroblasts

CAT - Cysteine Aminotransferase

CBS - Cystathionine-β-synthase

CDO - Cysteine Deoxygenase

CSE - Cystathionine-Y-lyase

CYP 450 - Cytochromes P450

Cys - L-Cysteine

D

DAPI - 4'-6-Diamidino-2-Phenylindole

DMEM - Dulbecco's Modified Essential Medium

DNA - Acid Deoxyribonucleic

DAXX- Death -domain associated protein

E

EAAT3 - Excitatory Amino Acid Transporter, member 3

EDTA - Trypsin- Ethylenediamine Tetra-acetic Acid

EGFR - Epidermal Growth Factor Receptor

ERA – Erastin

F

FA - Fatty Acids

FBS - Fetal Bovine Serum

FITC - Fluorescein Isothiocyanate

G

GCL - Glutamate Cysteine Ligase

GCLC - Glutamate-Cysteine Ligase Catalytic subunit

GCLM - Glutamate-Cysteine Ligase Modifier subunit

γ -Glu-Cys - gamma-L-Glutamyl-l-Cysteine

GPx - Glutathione Peroxidases

GR - GSH Reductase

GSH - Glutathione

GSS - GSH Synthetase

GSSG - GSH disulphide

GST - Glutathione S-Transferase H

H

H_2O_2 - Hydrogen Peroxide

H_2S - Hydrogen Sulphide

HRP - Horse Raddish Peroxidase

I

IF – Immunofluorescence

K

KRAS - Kirsten Rat Sarcoma virus

M

MAPK - Mitogen-Activated Protein Kinase

MDR - Multidrug Resistance

MDR1 - Multidrug Resistance protein (MRP) 1

MpST - 3-Mercapto-pyruvate Sulphur Transferase

MVP - Major Vault Protein

N

NADH - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NaOH - Sodium Hydroxide

NFκB - Nuclear Factor Kappa B

NRF2 - Nuclear Factor Erythroid 2- P45 -Related Factor2

O

OXPHOS-oxidative phosphorylation

P

PNETs-Pancreatic Neuroendocrine Tumours

PBS - Phosphate-Buffered Saline

P-gp - P-glycoprotein

PI - Propidium Iodide

PPP - Pentose Phosphate Pathway

PS - Phosphatidyl Serine

PTEN - Phosphatase and Tensin homolog gene

R

RNA - Ribonucleic Acid

RIPA - Radio-Immunoprecipitation Assay

ROS - Reactive Oxygen Species

RT - room temperature

S

SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Se - Selenium

SeChry/SeC - Selenium containing Chrysin

SLC1A1 - Solute Carrier Family 1 member 1

SLC7A11 - Solute Carrier Family 7 member 11

SSZ – Sulfasalazine

T

TBS - Tris Buffered Saline

TCA - Trichloroacetic acid

X

TCA cycle - Tricarboxylic Acid cycle

TMZ - Temozolomide

U

UGT - UDP-glycosyltransferases

UGT1A1 - UDP-glycosyltransferase family 1, member 1

V

V - volume

VDACs - Voltage-Dependent Anion Channel

W

W – weight

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ABSTRACT

Cancer is characterised as a set of diseases that is involved in uncontrolled cell growth with the ability to invade or spread to the other part of the body. Carcinogenesis is recognized as a process through aggregation of genetic and epigenetic changes in normal cell that ultimately leading to unlimited growth proliferation and invasion. Pancreatic neuroendocrine tumour (PNET) is a rare tumour that arise from neuroendocrine gland, occurs in various part of the body. The prevalence rate of PNETs is near about 25–30 per 100,000 population in the United States and according to Surveillance Epidemiology and End Results (SEER), the incidence rate of PNETs increased five-fold from 1973 to 2011. PNETs comprises approximately 7% of all types of cancer in the pancreas. Normally, 5 years survival rate of PNETs near about 42%.

PNETs is a heterogenous group of disorder with less 5 years survival rate due to lack of effective therapeutic options for patients with advanced stages, absence of symptomatology specially in case of non-functional PNETs and also to the phenomenon of chemoresistance, dependent on multiple mechanisms. Recent data shows that incidence rate of this tumour increases as a result of germline genetic mutations. Concerning to genetic change, it is very important to explain the differences that occurs at the level of chemoresistance. The treatment plan of the PNETs varies on type, location and aggressiveness of the tumour. Surgery is the only curative treatment in early stage but in advanced stages chemotherapy and radiotherapy are the most palliative treatment option of PNETs. Chemotherapy which is mainly based cisplatin combined with capecitabine and the response rate of treatment is near about 30%. Cisplatin is responsible for the formation of DNA adducts, leading to DNA damage, and induces generation of ROS, that consequently leads to oxidative stress, cell damage and death. Glutathione (GSH) plays an important role in the maintenance of intercellular redox balance and detoxification. Chemoresistance can be based on the alteration of the detoxification mechanisms and GSH system has been pointed as one of the most important. Cysteine is a rate limitant substrate for GSH synthesis, and xCT cyst(e)ine transporter is implicated in cancer severity and chemoresistance.

The **Hypothesis** of the project is: the disruption of xCT and uptake of cysteine leads to the reversal of resistance to alkylating agents in pancreatic neuroendocrine tumours (PNETs).

To accomplish the hypothesis we defined 3 aims: **1st aim** will be to address the expression of xCT in PNETs cell lines, and the modulation of xCT expression by cysteine and cisplatin; 2nd **aim** will be focused on the effect of xCT inhibition in PNETs cell death, using erastin and sulfasalazine, and **3rd aim** will be focused on the effect of new nanoformulations in order to disturb cysteine uptake (Sechry and Sechry@PURE$_{G4}$-FA) and glutathione synthesis (BSO@PURE$_{G4}$-FA).

Our work allowed to reveal the role of xCT transporter and the role of cysteine in PNETs cell line resistance. This cell line showing different response patterns in cysteine transporters activity helped to reveal the differences of the transporter in chemoresistance mechanism. It also showed that besides xCT transporter other cysteine transporter such as EAAT3 also appeared to be involved in the dynamics of chemoresistance mechanism.

This work was also important to undercover the effect of new nanoformulations in order to disturb cysteine uptake by using SeChry and GSH synthesis by using BSO in PNETs cell lines. SeChry, but not SeChry@PURE$_{G4}$-FA, induced cell death in BON-1 cell lines. SeChry cytotoxicity can be selective for cancer cells and this was taken in consideration in our new strategy by using SeChry@PURE$_{G4}$-FA, however the assay was not successful and new markers for targeted delivery must be investigated in PNETs.

BSO@PURE$_{G4}$-FA induced cell death in combination with platinum salts in PNETs cell lines. Possibly, the use of folate functionalised particles will help to bypass the critical step in the non-specific delivery of BSO to non-cancer cell. The targeted BSO delivery to cancer cells can be explored as a novel strategy in cancer therapeutics.

Moreover, more assays with cancer and non-cancer cells must be done in order to determine if folate receptor is in fact a suitable target to delivery drugs to PNETS cells, and find new and more specific targets.

Key-Words: Pancreatic neuroendocrine tumours (PNETs), platinum drugs, GSH, cysteine, xCT, , SeChry, Sechry@PURE$_{G4}$-FABSO, BSO@PURE$_{G4}$-FA.

RESUMO

O cancro é caracterizado como um conjunto de doenças envolvidas no crescimento descontrolado das células, com capacidade de invadir ou se espalhar para a outras partes do corpo. A carcinogénese é reconhecida como um processo de agregação de alterações genéticas e epigenéticas nas células normais levando ao aumento da proliferação e invasão. O tumor neuroendócrino pancreático (PNET) é um tumor raro que surge da glândula neuroendócrina e ocorre em várias partes do corpo. A taxa de prevalência de PNETs é de cerca de 25 a 30 por 100.000 habitantes nos Estados Unidos e, de acordo com a Surveillance Epidemiology and End Results (SEER), a taxa de incidência de PNETs aumentou cinco vezes entre 1973 e 2011. Os PNETs representam aproximadamente 7% de todos os tipos de cancro no pâncreas. Normalmente, a taxa de sobrevida dos PNETs em 5 anos é de cerca de 42%.

Os PNETs são um grupo heterogéneo de distúrbios com sobrevida inferior a 5 anos, devido à falta de opções terapêuticas efetivas para doentes com estágios avançados, ausência de sintomatologia especialmente no caso de PNETs não funcionais e também ao fenómeno de quimiorresistência, dependente de múltiplos mecanismos. Dados recentes mostram que a taxa de incidência deste tumor aumenta como resultado de mutações genéticas na linha germinativa. No que diz respeito às alterações genéticas, é muito importante explicar as diferenças que ocorrem no nível de quimiorresistência. O plano de tratamento dos PNETs varia de acordo com o tipo, localização e agressividade do tumor. A cirurgia é o único tratamento em estágio inicial, mas em estágios avançados a quimioterapia e a radioterapia são a opção de tratamento mais paliativo dos PNETs. A quimioterapia que é principalmente baseada em cisplatina combinada com capecitabina tem uma taxa de resposta de tratamento aproximadamente de 30%. A cisplatina é responsável pela formação de adutos de DNA, levando a danos no DNA, e induz a geração de ROS (espécies reativas de oxigénio), que consequentemente leva ao stresse oxidativo, danos celulares e morte. A glutationa (GSH) desempenha um papel importante na manutenção do equilíbrio redox intercelular e na desintoxicação. A quimiorresistência pode ser baseada na alteração dos mecanismos de desintoxicação e o sistema GSH tem sido apontado como um dos mais importantes. A cisteína é um substrato limitante da taxa para a síntese de GSH, e o transportador de cisteína xCT está implicado na gravidade do cancro e na quimiorresistência

A **hipótese** do projeto é: a interrupção do xCT e a captação de cisteína levam à reversão da resistência aos agentes alquilantes nos tumores neuroendócrinos pancreáticos (PNETs).

Para testar a hipótese, definimos três objetivos: o **primeiro** objetivo será abordar a expressão de xCT nas linhas celulares de PNETs e a modulação da expressão de xCT por cisteína e cisplatina; O

segundo objetivo será focado no efeito da inibição do xCT na morte celular de PNETs, usando erastina e sulfassalazina, e o terceiro objetivo será focado no efeito de novas nanoformulações para perturbar a captação de cisteína (Sechry e Sechry @ PUREG4-FA) e a síntese de glutationa (BSO @ PUREG4-FA).

Este trabalho permitiu revelar o papel do transportador xCT e o papel da cisteína na resistência das linhas celulares de PNETs. Estas linhas celulares mostrando diferentes padrões de resposta na atividade dos transportadores de cisteína revelaram as diferença no mecanismo de quimiorresistência e mostraram que, além do transportador xCT, outro transportador de cisteína, como o EAAT3, também poderá estar envolvido na dinâmica do mecanismo de quimiorresistência.

Este trabalho também foi importante para desvendar o efeito de novas nanoformulações, a fim de perturbar a captação de cisteína usando a síntese de SeChry e GSH usando BSO nas linhas celulares de PNETs. SeChry, mas não SeChry @ PUREG4-FA, induziu a morte celular em linhas celulares BON-1. A citotoxicidade de SeChry pode ser seletiva para células cancerígenas e isso foi levado em consideração na nossa nova estratégia usando o SeChry @ PUREG4-FA; no entanto, o ensaio não foi bem-sucedido e novos marcadores para entrega direcionada devem ser investigados nos PNETs.

BSO @ PUREG4-FA em combinação com sais de platina nas linhas celulares de PNETs induziu morte celular. Possivelmente, o uso de partículas funcionalizadas com folato ajudará a contornar a etapa crítica na entrega inespecífica de BSO às células não cancerígenas. A entrega direcionada de BSO às células cancerígenas pode ser explorada como uma nova estratégia na terapêutica do cancro.

Além disso, mais ensaios com células cancerígenas e não cancerígenas devem ser feitos para determinar se o receptor de folato é de facto um alvo adequado para administrar drogas às células de PNETs e encontrar novos e mais específicos alvos.

Palavras-chave: Tumores neuroendócrinos pancreáticos (PNETs), drogas de platina, GSH, cisteína, xCT, SeChry, Sechry @ PUREG4-FABSO, BSO @ PUREG4-FA.

1.INTRODUCTION

1.1 Cancer Biology:

The word cancer came from Greek, karkinos discovered by physician Hippocrates (460–370 B.C) to elucidate the carcinogenesis.[1] Cancer can be defined as a group of diseases characterised by abnormal cell division and alteration of normal cell behaviour leading to limitless growth of cell and proliferation.[1] In the developed countries, cancer remains the second leading cause of death, underlying approximately 10,000,000 deaths around the world.[2]

Cancer has some specific biological capabilities, justifying the complexity of neoplastic disease, called "Hallmarks" of cancer. The hallmarks of cancer are: sustained proliferative growth signals; evasion of antigrowth signalling; resisting programmed cell death; immense replicative capacity; induced angiogenesis, and capacity of cancer cell spreading - tissue invasion and metastasis.[3] Reprogramming of energy metabolism and evading immune destruction were added as emerging hallmarks of cancer in the last decade .[3]

In 1956, Otto Warburg established for the first time a connection between alterations in metabolism and cancer cells. He stated that a higher aerobic glycolysis was a distinctive feature of non-cancer and cancer cells, known as the Warburg effect.[4,5,6,7] Other evidence revealed that continuous aerobic glycolysis helps the cancer cell to activate oncogenes.[6,7,8] Additionally, the cancer cells grow like a mass with low amounts of nutrient and oxygen that prompt the growth of new vessels that helps the tumour to proliferate rapidly.[9]

The cancer cell microenvironment gives signals that lay into the activation of signalling pathways, which ultimately activate transcription factors. Besides cancer cells, stromal or non-malignant cells, permitting the mesenchymal phenotype that is necessary to invasion and metastasis distance; also contribute for a specifically required cancer cells transcriptional program.[10] Upon metastasis, cancer cells turn off this program and rescue an epithelial phenotype. It is thought that tumour microenvironment is playing a crucial role in this process.

1.2 Neuroendocrine tumours:

In 1969, Pearse identified APUD (amine precursor uptake and decarboxylation) peptide hormone secreting cells in the body that share common morphological features for balancing the embryological differences.[11] APUD cells have autocrine, paracrine and neuromodulator function, they are located throughout the body and mostly originated from neural crest.[12]
The main function of the neuroendocrine cells is to synthetize hormone in the cytoplasm then transport it to axons and from the nerve ending ultimately it is secreted into the vessels.[13]

Neuroendocrine tumours (NETs), characterized as rare tumours that arise from neuroendocrine cells, can occur in various organs of the body, including pancreas, gastrointestinal tract and lung.[14] The main origin of neuroendocrine cells is not well known. These cells have the behaviour of nerve cells and endocrine cells as they secrete hormones into the blood as a response to signals

that come from nervous system.[15,16] NETs which stereotypically ascend in the pancreas are called islet cell tumours.[15,16] NETs can be benign or malignant and they are usually slow growing, but sometimes they grow abruptly.
Symptoms and signs of the NET depend on some factors such as the types of the tumour, the size of the tumour, location of the tumour, the pattern *in situ* or spread and functioning state of the tumour such as hormone or non-hormone producing cells.[15,16] Detection and progression of NETs depend also on the traits of the neuroendocrine cells, because functional NETs may overproduce certain hormones inducing a constellation of symptoms related to the hormone that is being oversecreted, whereas non-functional NETs are not associated to a specific clinical syndrome .[17]
Most of the NETs are sporadic whereas some of these tumours are diagnosed in the context of to autosomal dominant hereditary cancer syndromes, such as: multiple endocrine neoplasia type 1 (MEN-1); Von-Hippel–Lindau; tuberous sclerosis and neurofibromatosis type 1.[18]

Treatment of NETs varies, as it depends on type, stage and aggressiveness of the tumour.[19] Therapeutic approaches, such as surgery and medical therapies may differ between each type of NET.

- Neuroendocrine tumour cells can be divided into aggregations of cells (glands) and diffusely distributed dispersed cells (disseminated system) and both differ from one another embryologically. [20]

Data from the Surveillance Epidemiology and End Results programme (SEER) including patients with NETs from 1937 to 2012 shows that the incidence of neuroendocrine tumours has markedly increased over past few years.[21]

1.2.1 Pancreatic Neuroendocrine Tumours

Epidemiology:

Pancreatic neuroendocrine tumours (PNETs) are a subtype of neuroendocrine tumour derived from the islet of Langerhans cells with various morphologies and behaviours, including malignant potential.[22]
In the United States, the prevalence of PNETs is approximately 25-30 per 100,000 population.[30] It is reported that the incidence rate of PNETs according to the population based report from Europe and Asia is near about <1 per 100 000 persons per year .[23,24,25] In addition, their incidence in autopsy studies is higher compared to diagnosed cases, ranging from 0.8% to 10%.[26] PNETS can be functional or non-functional. Functional PNETs are characterised by a hormonal hypersecretion syndrome such as hypoglycaemia, hyperglycaemia, glucose intolerance, peptic ulceration, watery diarrhoea, hypokalaemia etc. Non- functional PNETs do not produce any symptoms, but they cause illness and death by occupying the normal tissue and metastasizing.[27,28,29] Epidemiology of the PNETs is not well known and there is no difference of epidemiology based on sex, race, geographic area or economic status.[30]

Biology and Genetics:

In 2000, the World Health Organization (WHO) histologically classified GEP-NENs as: well differentiated endocrine carcinoma and poorly differentiated endocrine carcinoma/small cell carcinoma. However, in 2010 they categorized the Classification based on Ki-67 proliferation index and mitotic count, as: neuroendocrine tumour grade 1 (NET-G1); neuroendocrine tumour grade 2 (NET-G2), and neuroendocrine carcinoma.[31] Mitotic count ranges from <2 to >29 per HPF (high power field) and Ki-67 proliferation index <3% to >20% per HPF according to the grade of tumour.[31]

Genetic information provides a better knowledge for the development of new treatment agent in the field of research. Genetic differentiation between the different grades of tumour in PNETs also gives us useful information to better understand the biology of PNETs. In 2009 Capelli et al. reported *MEN1* gene mutation as the most common hereditary irregularity in the germline PNETs causing biallelic inactivation due to the fact that a mutation in one allele induces the loss of the second allele. *MEN1* gene is a tumour suppressor gene and its germline mutation influences the development of multiple endocrine neoplasia syndrome type 1.[32] Mutations in *ATRX* (α-thalassemia/mental retardation syndrome x-linked) and *DAXX (*Death -domain associated protein) genes are reported as the most common somatic events involved in PNETs. Both genes are associated with chromatin remodelling in the telomeres and the encoded proteins of the genes are networked with one another.[33] In addition, based on the molecular pathway of poorly differentiated PNETs, cancer cells can be derived from the normal neuroendocrine cells which acquired mutations of *TP53* and *SMAD4* concomitant with *KRAS* mutations.[34]

Therapy:

The therapeutic approach for the PNETs patients differs, depending on the type, location, stages and aggressiveness of the tumour. Generally, it is divided into three levels, such as surgery, anti-proliferative and symptomatic therapies.[34]

Imaging studies are used to evaluate the extent and the location of the tumour, so that proper management can be taken.[35] From past to present day, surgery with regional lymph node resection is the only curative treatment recommended for the early stage PNETs and is specially used to prevent complications in PNET patients.[36] However, in advanced stages, surgery is usually not feasible and systemic therapy becomes the main treatment option. However, in patients with advanced PNETs and liver metastasis, partial hepatectomy may be recommended, depending on the patient response, size and location of primary tumour.[36] Chemotherapy and radiotherapy are the most common palliative approaches to advanced stage patients, when the liver is not resected.[37]

1.3 Chemotherapeutic agents:

Platinum based chemotherapy is the most frequently used chemotherapeutic strategy. Platinum drugs such as cisplatin, carboplatin or oxaliplatin are commonly combined with taxanes (paclitaxel or docetaxel). Cisplatin is a first line chemotherapeutic agent with the highest side effects. Carboplatin is the second-generation antineoplastic agent containing platinum. The main differences between carboplatin and cisplatin are that carboplatin has a cyclobutanedicarboxylate group, being more stable but to achieve the same effect as cisplatin this drug need to be applied in a higher concentration. Within this group of drugs, carboplatin is the best option as a neoadjuvant, adjuvant and palliative treatment because of its less side effects.[38,39]

Chemotherapy is the treatment for the advanced stage with unresectable pancreas tumour, resulting in an increase in overall survival of patients.[40] When a patient shows symptomatic features and/or has a tumour with a ki-67 value more than 10%, cytotoxic chemotherapy is well-thought-out as a first line of treatment. Two different groups of medications are utilized for the treatment purpose of well differentiated PNETs. They are alkylating agents such as platinum salts, Streptozocin (STZ), temozolomide (TEM), dacarbazine (DTIC) and antimetabolite agents such as 5-flurouracil (5FU), capecitabine (CAP) and doxorubicin (DOX). Nowadays, combination of STZ and 5FU is prescribed commonly instead of STZ and DOX combination. The limitation of use of DOX is due to cardiotoxicity with a dose of 50 mg/m^2, as reported.[41] STZ is a diabetogenic substance, which is selectively incorporated into the beta cells of the pancreas via GLUT2 transporters that leads to cytotoxicity and the generation of reactive oxygen species (ROS). On the other hand, another combination of drugs, TEM combined with CAP, is recommended as an alternative treatment in well differentiated PNETs patients. In advanced cases of neuroendocrine tumours / neuroendocrine carcinomas, cisplatin-based chemotherapy is standard (generally, cisplatin and etoposide).[41]

1.4 Chemoresistance:

Chemoresistance is one of the most challenging issues in the field of cancer treatment. Consequently, to find out novel therapeutic approaches it is vital to know the molecular mechanisms of the chemoresistance, because it causes recurrence, relapse and death.[42] Chemoresistance can be divided into two categories, one is intrinsic (*de novo* or innate) and another is acquired resistance.[43]

Intrinsic resistance is characterized as a circumstance when chemotherapy is not enough from the beginning of the treatment because of the patient endogenous component, on the other hand, when the tumour grows slowly after exposure of anticancer medications, because of epigenetic or molecular adjustment of malignant cells, it is known as acquired resistance.[44] There are many diverse mechanisms involved in chemoresistance such as alterations of drug transport pump, modification of drug target interaction, enhanced DNA repair activity, augmented drug damage tolerance, effect on protein associated with detoxification process and imperfection in

apoptotic pathway.[45] In advanced stages of pancreatic cancer, chemotherapy is the most effective treatment. Nevertheless, many patients lean towards the relapse of disease and chemoresistance.[46]

To understand the chemoresistance mechanism related to **transport pumps**, it is important to focus on p-glycoprotein. **P-glycoproteins** are membrane proteins, including Multidrug resistance protein 1 (MDR1) or ATP binding Cassette (ABC) protein, acting as an ATP -dependent efflux pump. These proteins cause decreased drug intracellular accumulation by altering the intracellular pH.[38] There are some other transporters such as Breast Cancer Resistance Protein (BCRP) and Major Vault Protein (MVP). BCRP bearing circulating microvesicles contribute to the mechanism of chemoresistance in breast cancer.[47] MVP possibly will facilitate chemoresistance by controlling the nucleo-cytoplasmic transport (hormones, ribosomal RNAs, drugs).[38]

Modification of drug target expression or mutation causes alterations in drug targeting that ultimately leads to drug resistance in cancer. For example, a mutation in the topoisomerase II gene leads to reduced efficacy of topoisomerase inhibitors, affecting DNA synthesis, DNA damage and halting of mitotic processes. Some anticancer drugs target signalling kinases such as epidermal growth factor receptor (EGFR), Ras, Src, Raf, and MEK. In addition, some of these kinases are constantly active in certain types of cancer, leading to uncontrolled cell growth.[48]

In the field of cancer drug resistance, **enhanced DNA repair and tolerance of damaged DNA are** thought to be one of the possible mechanisms of chemoresistance. The role of chemotherapeutic agents is to damage the DNA of cancer cells indirectly or directly and DNA repair mechanisms fix the damage of the DNA[49]. It is known that platinum based chemotherapeutic agents cause apoptosis of cancer cell through damaging the DNA.[50]

It is thought that polymerase enzymes play an important role in the process of drug tolerance. The normal DNA replication process requires the action of error free DNA polymerase. Moreover, when DNA injury cannot be repaired, in order to sustain the cell survival, the injury should be tolerated, and this can be accomplished by translational synthesis pathway catalysed by DNA Polymerase.[51]

Drug damage tolerance is associated with reduced susceptibility to apoptosis. Catalytic caspases, which are associated to a group of cysteine-dependent aspartate-directed proteases, have their activity dependent on the formation of tetrahedral intermediates via promoting a cysteine residue that is linked with apoptotic activity and signalling. In addition, genes that regulate apoptosis are altered in this mechanism, accounting for apoptosis inhibition.[52,53]

Effect on proteins associated with the detoxification process, as glutathione and its related enzymes, is another chemoresistance mechanism. Glutathione plays an important role in the process of detoxification. Glutathione-s-transferase (GST) superfamily network is an important example of drug activation and inactivation process. GST contributes for the progress of drug resistance mechanism by direct detoxification and through inhibiting the pathway of mitogen activated protein kinase (MAPK).[48] The higher GSH level with over expression of glutathione-s-

transferase (GST) will increase the proportion of conjugation and detoxification reduces the effectiveness of therapeutic agents.[54] Cytochrome P450 (CYP) is also involved in the process of drug activation and inactivation. CYP is divided into two classes: Class I includes CYP1A1, CYP1A2, CYP2E1, CYP3A4 and Class II includes CYP2B6, CYP2C9, CYP2C19, CYP2D6. Class II has a characteristic feature of high polymorphism rate that is involved in the metabolism of cancer drugs and in drug resistance mechanism.[48] Uridine diphosphate- glucuronosyltransferase (UGT) superfamily, which is a group of transferase enzymes involved in glucuronidation catalysis, regulating the formation of inactive hydrophilic glucuronides with substrates together with environmental carcinogens and cytotoxic. The expression of UDP glycosyltransferase family -1, member 1 (UGT1A1) thought to be negatively regulated by DNA methylation meanwhile due to epigenetic changes the overexpression of UGT1A1 is common in cancerous state, that ultimately leads to drug detoxification.[41]

Programmed cell death is markedly influenced by a variety of genes, some of them are mutated or difunctionally regulated. Defective cell death can occur as a result of aberrant regulation of TP53 and PTEN, inhibitors of the PI3K/AKT pathway, which might impair the affiliation between DNA damage and the activation of programmed cell death, increasing chemoresistance and leading to drug induced apoptosis failure.[54] *TP53* inhibits *PI3K* ultimately abolishing the mitochondrial p53 -dependent apoptosis. PTEN is an inhibitor of PI3K for the synthesis of PIP3 by phosphorylation, which will activate AKT. When PTEN in downregulated or inhibited, PI3K will be activated, PIP3 will be synthesized and AKT activated.[55,56] Apoptosis is activated by the cleavage of executioner caspases (eg. 3 or 7). There are two routes to apoptosis: extrinsic (or death receptors) and intrinsic or (mitochondrial).[57,58] Extrinsic pathway is regulated by the action of FAS and TNF-α on the death receptors and it is mainly dependent on caspase 8 activation that will cleave the executioner caspases. Within the external pathway mutations, epigenetic or post-translational alterations may affect proteins function, which ultimately lead to the inhibition of cell death in cancer cells.[57,58,59] Within the intrinsic pathway the up regulation of the anti-apoptotic genes (eg. Bcl2) and down regulation of pro apoptotic genes (eg. Bax, Bcl_{xl}) in tumour cells lead to resistance to chemotherapeutic agents.[60] XIAP is also an inhibitor of caspases 3, 7 and 9, affecting both apoptosis pathways.[57]TP53 is the master regulator of apoptosis, being an activator of both intrinsic and extrinsic pathways, in cancer deregulation and mutation of TP53 affects apoptosis through the inhibition of mitochondrial and death receptor signalling. [57,61]

1.5 Cancer metabolism.

The basic principle of cancer metabolism is that metabolic activities are changed in the cancer cells compared to normal cells. In 2011, Weinberg et. al. added a new hallmark of cancer that is reprogramming of cancer metabolism. The metabolic reprogramming has a reflective effect on gene expression, cellular proliferation and tumour microenvironment.[54] Otto Warburg in 1923, first made the connection between cancer and metabolism in the basis of the concept of Warburg effect, assuming that cancer cells would preferentially fulfil glycolysis instead of oxidative

phosphorylation (OXPHOS).[62] Metabolism not only is related to intracellular network, but is also related with extracellular organic and communication molecules, which control the complete metabolic function of the cell. Fibroblasts are the most important type of tumour stromal cells, playing a vital role in the process of cancer cell progression through their molecular cooperation.[63] CAFs (cancer associated fibroblasts) and neoplasm cells permit an enhanced microenvironment, vital for neoplasm survival, proliferation, migration and chemoresistance.[64,65] CAFs are crucial in the metabolic process to organize the inner source of nutrients providing the Krebs cycle with metabolic intermediates.[66] Fatty acids are major contributors for tricarboxylic acid (TCA) cycle, that is required for the generation of nicotinamide adenine dinucleotide (NADH), vital for OXPHOS.[63] Reactive oxygen species (ROS) are short lived molecules with unpaired electrons that are constantly generated, modified and eliminated during the cellular processes together with metabolism, proliferation, differentiation, processes of immune system regulation and remodelling of vascular system; but the excess amount of ROS causes oxidative stress and altered cancer metabolism.[67] Excess production of ROS causes alterations of sulfhydryl groups of private kinase M2 (PKM2) leading to shunting of glucose away from glycolysis towards the pentose phosphate pathway (PPP), which produces NADPH and nucleotides, favouring cell proliferation.[72] The NADPH reduces glutathione into an active antioxidant, functioning as a cell protector and maintain the redox homeostasis.[68,69]

1.5.1 GSH and Cysteine:

Glutathione (GSH) and cysteine are both thiols, having a sulfhydryl or thiol group, made of sulphur and hydrogen atoms attached to a carbon. GSH is a low molecular weight thiol composed of glutamic acid, cysteine and glycine. GSH is very reactive and can conjugate with another molecule via sulfhydryl group. The bulk of GSH found intracellularly within the cytoplasm is approximately 90%, about 10% is found in mitochondria and a little amount is found in the endoplasmic reticulum.[70] GSH is synthesized by two ATP requiring enzymes glutamate cysteine ligase (GCL) and GSH synthetase (GSS). The synthesis of GSH is catalysed by GCL which have two subunits, one is GCLC (glutamate cysteine ligase catalytic subunit) that catalyses the linkage between the amine group of cysteine and the γ-carboxyl group of glutamic acid; and another is GCLM (glutamate cysteine ligase modifier) that increases the catalytic activity by interacting with GCLC. GSH synthetase catalyses the reduction of gamma-glutamylcystein and glycine to create GSH.[71] Then this gamma-glutamylcystein can be transported back into the cells and again metabolized into 5-oxoproline, ultimately converted to glutamate and used in GSH synthesis. The rate of GSH synthesis largely depends on cysteine availability and the activity of GCL. Cysteine plays a key role in the maintenance of protein structure and synthesis. The proper concentration of intracellular cysteine also helps to maintain the redox homeostasis.[72]

GSH is the most reduced form, which is being oxidized in the form of GSSG via its direct reaction with ROS or substitute as a co-enzyme for antioxidant like glutathione peroxidase (GPX) and recycled by glutathione reductase (GR).[73,74,75] GPX is the member of the family of selenium -

dependent enzymes, which can differ from transferases because of its activation with hydrogen peroxide (H_2O_2). GPX enzymes use glutathione like a ROS scavenger, that convert H_2O_2 to water and lipid peroxides.[75,76] Glutathione plays an important role in the maintenance of redox balance and protein status. The glutathione reaction with the protein varies with the concentration of GSH and GSSG. But the reversible thiolation reaction of proteins is responsible for the regulation of numerous metabolic processes such as enzymatic activity, signal transduction and gene expression with the help of redox sensitive nuclear transcription factor like AP-1, NF- kappa B and TP53.[77,78]

Another important function of GSH is the storage of cysteine because it is unstable extracellularly and rapidly oxidized to cystine through a process that produces toxic ROS. The γ-glutamyl cycle helps GSH in this procedure of releasing cysteine. In this cycle, GGT transfers the γ-glutamyl to an amino acid to form γ-glutamyl-aa and cysteinyl-glycine. GSH plays an important role not only in the protection of cells against apoptosis but it also has an extreme key role in detoxification process, and its disruption will activate diverse transcription factors like activator protein -1 (AP-1), activator protein -2 (AP-2), stress activated protein kinase (SAPK) and c-Jun-N -terminal kinase (JNK). Besides, GSH acts as an antioxidant .[71,79], it is also involved in DNA repair and synthesis and prostaglandin synthesis.[80] Increased levels of GSH are considered as a causative factor of drug resistance through binding to or interacting with the drug or ROS, preventing harm to protein or DNA or by taking part in the DNA repair process.[68] However, specific GSTs overexpression can even influence chemoresistance, while polymorphisms which reduce GST activity are related to a high risk of developing cancer.[73] In combination, it will also increase the percentage of conjugation and detoxification of chemotherapeutic agents, that ultimately reduces their effectiveness .[81] Higher expression of GST can switch the balance of kinases that will cause a potential benefit for cancer development.[73]

1.5.1.1 Cysteine metabolism

Cysteine is a semi essential amino acid which is mainly provided through diet or through the trans-sulphuration pathway. Serine and methionine-derived homocysteine reaction are catalysed through cystathionine-β-synthase (CBS) to form cystathionine, then the pathway is facilitated by cystathionine-γ-lyase (CSE) and catalyses its final step through the conversion of L-cystathionine to cysteine and α-ketobutyrate. [82]

Cysteine degradation is also a crucial metabolic reaction. Multienzymes are involved in this procedure. The process of sulfane sulphur generation from cysteine, homocysteine and their disulphides is carried out through three enzymes that are CBS, CSE and 3-mercapto-pyruvate sulphur transferase (MpST) along with cysteine aminotransferase (CAT). CSE is responsible for catalysing the conversion of L-cystine to thiocysteine, pyruvate, and ammonia. After that, thiocysteine in the presence of thiol forms cysteine and inorganic sulphur from hydrogen sulphide (H2S) and cystine.[83] MpST enzyme is responsible for the relocation of sulphur ion from 3-mercaptopyruvate toward a thiolic compound.[84] MpST and CAT enzymes are in mitochondria

and are responsible for mitochondrial ATP production dependent on H_2S, while CBS and CSE are repositioned in mitochondria under cellular stress.[85] L-cysteine is also degraded by the help of cysteine dioxygenase (CDO). Cysteine dioxygenase is accountable for adding an oxygen molecule to sulphur of cysteine, which is converted to sulfinic acid, known as cysteine-sulfinic acid. After that, cysteine-sulfinic acid is again metabolised into either one pyruvate and sulfate or to taurine and CO_2.[86]

It has been reported through literature that cysteine is a tumorigenic promoter. Pancreatic cancer cells need an exogenous source of cysteine because of its crucial role in redox homeostasis contributing for tumour growth and maintenance. Cancer cells depend on oxidized cysteine, which detoxifies the lipid ROS and prevents ferroptosis. [87]

1.5.1.2 Cyst(e)ine /Glutamate transporter: Cysteine transportation is mediated by alanine - serine-cysteine transport system (ASCT), excitatory amino acid transporters (EAATs) system and the Xc^- (xCT) system. ASCT system includes ASCT1 and 2, in which Na^+ dependent mediated transport exchanges small neutral amino acids like alanine, serine, threonine and cysteine and structure of the transport. EAATs mediate the transport of amino acids along with the co - transport of K^+ via producing a net flux of charges. xC^- system depends on xCT transporter, a glutamate and cystine (the oxidized form of cysteine) antiporter that relies on proton electrochemical gradient.[88,89]

1.5.1.2.1 System xC^-(xCT): System xC^- was first explained by Bannai and Kitamura in 1980 in culture of human fetal lung fibroblasts, which work as a sodium independent and chloride dependent antiporter system, being capable of transporting an anionic form of cysteine and glutamate in both directions.[90,91,92] Cystine is the oxidised form of cysteine, which is rapidly reduced and therefore the intracellular glutamate concentration is mostly elevated comparing with the extracellular space, this mechanism basically relies on the concomitant cysteine import and glutamate export.[93]

The xCT transporter is encoded by solute carrier family 7-member 11 (*SLC7A11*) gene, located on chromosome 4 (4q28.3). *xCT/SLC7A11* gene has an antioxidant responsive element region (ARE) in its promoter that is controlled by the regulation of nuclear factor erythroid 2-related factor 2 (NRF2), which plays an important role in cellular protection against oxidative stress. In 2018, Carpi-Santos et al. found that alterations in xCT expression affected the retina of diabetic rats via regulation of NRF2.[94,95] Meizi et.al made an experiment with the aim of making a link between sepsis illness and protein interaction with C-kinase 1 (PICK1). Through this experiment they found that deficiency of PICK1 not only causes the inhibition of xCT expression, but also reduces the GSH synthesis, which ultimately leads to severe oxidative stress.[96] xCT activity also has a role in cancer. In invasive breast cancer and oesophageal squamous cell carcinomas, expression of xCT is negatively correlated with survival.[97] Besides overall function, it is also reported that system

xCT also plays an important role in the immune system. xCT overall acts as an anti-oxidant, it contributes for cysteine uptake and GSH synthesis, ultimately solving the oxidative stress.[88,98]

xCT transporter is made of a light chain (xCT) and a heavy chain (4F2hc) and both chains are connected through a disulphide bridge. However, the heavy chain is responsible to link with specific amino acids of the light chain to form a heterodimer.[74,88,99] In the cell membrane, xCT is necessary to uptake the enough cysteine that is required for the GSH synthesis, which acts as an antioxidant for the maintenance of intracellular redox balance.[93,100] xCT is not only highly expressed in a variety of cancer cells like breast cancer, prostate cancer, lymphoma and glioma, but it is also engaged in various cellular functions in cancer cells like chemoresistance.[101,102,103,104,105,106] It is known that neurotransmitter glutamate depends on xCT, so the role of xCT is well known in brain or central nervous system (CNS). It has been found that CNS dysfunction, accompanied by neural oxidative stress and accumulation of glutamate in the extracellular space, causes the reduction of cysteine import in the cell and increases the production of ROS which ultimately leads to excitotoxicity.[87] It is also observed that glioblastoma cells show higher expression of xCT.[88,107]

xCT transport system has a role in cancer context since it causes increased levels of GSH, involved in the process of chemoresistance.[108,109] But the inhibition of xCT function also interferes with its expression. Sulfasalazine and Erastin are well known inhibitors of this transport system.[108,110]

Sulfasalazine is a drug which is used in inflammatory bowel disease and rheumatoid arthritis. Approximately 90% of sulfasalazine reaches the colon and it is metabolized into sulfapyridine and mesalazine by the help of bacteria.[111] Recently, it has been appointed as an inhibitor of xCT, but the mechanism of action remains unclear.[88] A recent study found that sulfasalazine along with autophagy-inducing agent temozolomide produce higher efficacy of chemotherapy drug in glioma treatment.[112] It is known that caveolin-1 pathway is involved in the process of tumour metastasis. Some recent experimental data provided evidence that sulfasalazine by interfering with the pathway of caveolin-1 causes the reduction of GSH levels, which ultimately reduces tumour metastasis in oesophageal squamous cell carcinoma.[113] Data collected from current studies demonstrate that xCT has a major role in pancreatic cancer via enhancing the GSH synthesis, whereas sulfasalazine known as a nontoxic drug combined with gemcitabine initiates an effective treatment in pancreatic cancer. [114]

Erastin is a small molecule binding with voltage-dependent anion channels (VDAC) which are responsible for the initiation of iron dependent cell death known as ferroptosis. Erastin acts as an inducer of ferroptosis by reducing cysteine uptake and diminishing the synthesis of GSH, ultimately maintaining the redox homeostasis.[115] It was proven by some studies that RNA interference mediated knockdown of VDAC2 or VDAC3 causes resistance to Erastin.[116] VDAC2 or VDAC3, which are the two isoforms of VDAC are involved in inhibiting the cysteine/glutamate transporter system. In addition, Erastin can inhibit the activity of GPX-4, which is a GSH related enzyme giving rise to oxidative damage to cells.[117] It has a robust inhibitory effect on xCT activity compared to sulfasalazine. [118] In 2007, Yagoda. et al. recognized that Erastin showed evidence in

tumour cell death with harbouring mutations in RAS-RAF-MEK genes. They also found that this drug changed the permeability of the outer mitochondrial membrane, indicating that maybe Erastin induces apoptosis in cancer cells.[116]

1.6 New strategies to disturb xCT and cysteine /GSH metabolism by using selenium-chrysin (SeChry) and buthionine sulfoximine (BSO)

Selenium (Se), acts as a cofactor of mammalian enzymes, such as glutathione peroxidase, functioning as a great antioxidant.[119] It is known that cancer cells are more sensitive to Se induced cytotoxicity than non-cancer cells.[120,121] Se produces ROS and instigates DNA strand breaks with subsequent cell cycle arrest and apoptosis, leading to tumour growth suppression.[122,123] Pro-oxidative effects of inorganic Se compounds were attributed to the antitumor mechanism, instead of antioxidative effects of organic selenium compound.[124] Se is incorporated into selenoproteins and the link between selenoproteins and cancer depends on the anti-oxidant properties of selenoproteins including the enzymes of glutathione peroxidase (Gpx) family, responsible for antioxidant protection against ROS.[125] High ROS generation induces continuous oxidative stress that provides expression of malignant phenotype of cancer cells.[126] Se metabolite conjugates to two glutathione (GSH) moieties, known as selenodiglutathione (SDG), increasing intracellular accumulation of Se. Evidences suggest that SDG in Se incorporation proceeds at cell surface by gamma-glutamyl transpeptidase (GGT), prompting the creation of selenocysteine, which is probably going to be exported through xCT. This mechanism could be arbitrated by xCT and may cause the Se induced cancer specific toxicity.[119] As it is known, xCT transporter is related to cancer drug resistance, in an effort to reduce the detoxifying activity through increasing the uptake of cysteine, accounting for GSh synthesis. Hence, selenated-compounds were taking in thought as potential xCT inhibitors.[127,87] Selenium-chrysin (SeChry) is an organoselenium compound composed of a selenium group and chrysin, a flavone, having anti-tumoral and anti-oxidant properties, by protecting cells from toxic compounds and on the other hand, by inducing apoptosis.[128,129,130]

Buthionine sulfoximine (BSO) is a specific γ-glutamylcysteine inhibitor, developed by Griffith and Meister that reduced cellular GSH levels biosynthesis. BSO is a chiral compound and consists of two isomers, one is L-buthionine-(R)-sulfoximine and the other one is L-buthionine-(S)-sulfoximine. [131] L-buthionine-(S)-sulfoximine has a greater role in the inhibition of γ-GCS in relation to is L-buthionine-(R)-sulfoximine in cell culture and in animals.[132,133] It was utilized in numerous investigations to reveal a decrease in GSH, sensitizing both cell lines and animals.[134,135,136] As above-mentioned, GSH is the most commonly found intracellular thiol, its depletion by BSO prompts oxidative stress, ultimately leading to increased radiation sensitivity.[137] In addition, BSO sensitizes cancer cells to chemotherapy.[138,139] Preliminary studies from our group and studies from other teams revealed that continuous infusion of BSO give rise to the depletion of tumour GSH in patients with advanced cancers, specially ovarian cancer,[140,141] which prompt us to test this compound in PNETs .

Cancer cells express higher levels of folate receptor than normal cells [142,143] and folate receptor targeted therapy is a way of directing nanoparticles to cancer cells both *in vitro* and *in vivo.*[143,144] So, in an attempt to improve the specificity of SeChry and BSO towards cancer cells, folate receptor targeted delivery of these compounds was performed using SeChry@PURE$_{G4}$-FA nanoparticles as a drug delivery system.[145,146,147]

1.7 Metabolic profiles of PNET

Metabolic alterations in neoplastic cells is nowadays considered as a hallmark of cancer.[3] In addition, metabolic reprogramming accelerates the proliferation of cancer cells.[148] Most of the cancer cells depend on the amino acid glutamine, instead of glucose, to fulfil their biosynthesis need.[149] As it was previously explained that PNET can exhibit mutations of *TP53* with activating *KRAS* mutations, it is thought that *KRAS* driven metabolic alterations might have a prominent role in PNET proliferation. In pancreatic ductal adenocarcinoma, mutated tumour suppressor genes such as *TP53*, *SMAD4* have a significant role in the process of carcinogenesis.[150] It is proven that elevated glycolysis flux causes a boost up the downstream pathway of *KRAS*. In the context of cancer, mutated *KRAS* transcriptionally upregulates the GLUT1 that ultimately causes elevated glucose uptake.[151,152]In glycolysis, pyruvate is converted to lactate upon the action of lactate dehydrogenase enzyme (LDH). Mutated *KRAS* also has a role in increasing LDHA expression.[116]

1.7.1 Thiols (including GSH and Cysteine) metabolism in PNET: Thiols especially GSH interfere with complex tumorigenic process. GSH conjugation of electrophilic carcinogens may prevent tumour initiation and defend cells against oxidative stress. It is thought that enhancement of antioxidants or conjugating capacity by increasing the levels of GSH, via precursor application, synthesis stimulation or inducing the related enzymes, may constitute a chemo preventive cell strategy.[153]

GPX-1 (GPX-1), which is a member of GPX family, is known as an enzyme that is related to tumour initiation and progression via modulating intracellular ROS by eliminating hydroperoxides.[154,155,156] Interestingly, the relation between this enzyme and pancreatic cancer is not well known, even though it was already proven by some experimental work that GPX-1 expression is directly linked to the initiation and progression of several malignancies such as breast, bladder and prostate cancer.[157,158,159] It is thought that GPX-1 might have some role in pancreatic cancer, although showing some controversy, as it seems that the abrogation of GPX-1 is related to pro-metastatic pathways dependent on ROS.[160] However, in PNET there are no studies on the role of thiols and related enzymes in disease progression. ROS have double roles, as hydroperoxides they promote tumour proliferation, migration and the process of angiogenesis, whereas excessive production causes cell apoptosis.[161]

Dietary source is considered as an etiological factor for pancreatic cancer. Therefore, some studies already proved that deficiency of dietary sources of methyl group like choline, methionine, vitamin B-12 and folate have a direct role in pancreatic dysfunction, in addition these elements have a role in developing various types of cancer including pancreatic cancer. [162,163,164]

Folate has a methyl group, which is required for the intracellular methylation reaction. In folate metabolism methylene tetrahydrofolate reductase (MTHFR) catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the principle form of folate in the blood and acts as a co-substrate to form methionine from homocysteine. [165] Low levels of folate are associated with the incorporation of uracil instead of thymine into DNA, which ultimately elevate the risk of DNA mutations and DNA strand breakage.[166] Data collected from a case control study conducted by Shirisha et. al showed the that percentage of direct DNA damage was higher in pancreatic cancer patients comparing to healthy individuals. [167] In addition, folate pathway has an important role in the process of DNA synthesis and repair.[166] It is thought that altered folate metabolic pathway, might have some role in pancreatic cancer, as well as in PNET. Nevertheless, as far as we know, the knowledge about metabolic alterations in PNET, contributing for cancer progression and chemoresistance is scarce.

2. Hypothesis and Aims:

The **Hypothesis** of the project is: the disruption of xCT and uptake of cysteine leads to the reversal of resistance to alkylating agents in pancreatic neuroendocrine tumours (PNETs).

This thesis project has three main objectives:

First aim will be to address the expression of xCT in PNETs cell lines, and the modulation of xCT expression by cysteine and cisplatin.

Second aim will be focused on the effect of xCT inhibition in PNETs cell death, using erastin and sulfasalazine.

Third aim will be focused on the effect of new nanoformulations in order to disturb cysteine uptake (Sechry and Sechry@PURE$_{G4}$-FA) and glutathione synthesis (BSO@PURE$_{G4}$-FA).

3. Experimental procedures

3.1 Cell Culture

Two different pancreatic neuroendocrine tumours (PNETs) cell lines were used: BON-1 (CVCL_3985; JCRB cell Bank) and QGP-1 (CVCL_3143; JCRB cell Bank). BON-1 cell line was cultured in Dulbecco's Modified Essential Medium/Nutrient mixture F- 12 (Ham) 1x (DMEM F-12) (11330-032, Gibco, Life Technologies) and QGP -1 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium 1x (12-167F, Lonza, Bioscience), L-glutamine supplemented. Both mediums were supplemented with Fetal Bovine Serum 10% (FBS) (S 0615, Merck), Antibiotic-Antimycotic (AA) 1% (P06-07300, PAN Biotech) and Gentamicin 1% (15750-060, Gibco, Life Technologies). Cells were preserved under a temperature of 37^0C, 5% CO_2 in a humified environment. The cells were cultured until 75% -100% optimal confluence before they were detached with 0.05% Trypsin- Ethylenediamine Tetra-acetic Acid (EDTA) (25300-054, Invitrogen, Thermo Fisher Scientific) at room temperature for 5min. Cell number was determined using a Bürker counting chamber.

For cell viability analysis, 2 x 10^5 cells/mL were plated in 24 well-plates (500 µL/well) and for western blotting 2.5 x 10^5 cells/mL were plated in 6 well-plates (2 mL/well).

After 8 h of starvation (FBS free culture medium), cells were exposed to L-cysteine (0.402 mM); cisplatin (0.025 mg/mL), sulfasalazine (0.25 mM) and erastin (5 µM) isolated or in combination, in 1% FBS culture medium for 16 h.

In BON-1 data, a homozygous (AF 100%) g.114713908TOC mutation (ENSP00000358548 **p.Q61R**) was found in codon 61 in NRAS. Furthermore, a heterozygous g.25245350COA mutation (ENSP00000 308495 **p.G12V**) with an allelic ratio of 0.71 was originate in the KRAS gene in QGP 1. Data collected from (*Whole -exome characterization of pancreatic neuroendocrine tumour cell lines BON- 1 and QGP -1*).

3.2 Flow Cytometry -cell death analysis

To evaluate the effect of cysteine (0.402 mM), cisplatin (0.025 mg/mL), erastin (5 µM), sulfasalazine (0.25 mM) and BSO@PURE$_{G4}$-FA (456 µM) under (8h) starvation with 16h of experimental conditions and in SeChry@PURE$_{G4}$-FA (19 µM) without starvation with 24 h of experimental conditions in PNET cell lines, cell death analysis by flow cytometry was performed.

Flow Cytometry is a most commonly used technical method to measure the physical and chemical characteristics of cells. The most common typical feature of apoptotic cells is characterized by fragmentation and loss of nuclear DNA. Upon apoptosis the plasma membrane undergoes some structural changes and phosphatidyl serine (PS) is translocated onto the cell surface. Moreover, when the apoptotic process is started PS is translocated in the outer leaflet of the plasma membrane surface. Annexin V helps to identify the apoptotic cells by binding to PS. Red

fluorescent propidium iodide (PI) is also used to stain the necrotic cells. When the cell membrane is disrupted, it binds to double strand DNA by intercalating between base pairs, thus allowing PI entering into the cell and staining necrotic cells.[168,169,170]

Furthermore, cells were collected. Supernatant from each well was collected to an Eppendorf and adherent cells were harvested with 100 µL of 0.05 % trypsin -EDTA (25300-054, Invitrogen), after that cells were collected to the same Eppendorf, in which the supernatant was at first collected. This way, it was ensured that all the cells that were in suspension in culture medium and adherent were analysed. Cells were centrifuged at 255 × g at room temperature for 2 min. The supernatant was discarded and cells were washed with PBS 1X - 0.1% BSA and stained with 0.5 µL FITC- Annexin V (640906, Bio Legend) (100 µg/mL) in 100 µL annexin V binding buffer 1X for each sample and then incubated in a dark place at room temperature for 15 mins. After that samples were rinsed with 100 µL PBS (1X) -0.1% of BSA, and centrifuged at 255 × g for 2 min. Cells were resuspended in 200 µL of annexin V binding buffer 1X 10 mM Hepes (pH 7.4), 0.14 M sodium chloride (NaCl), 2.5 mM calcium chloride ($CaCl_2$). 2.5 µL of propidium iodide (PI; 50 µL/mL) were added to all the samples, 5 min prior the acquisition in a FACS calibur (Becton Dickinson). Flow cytometry data was analysed by Flow Jo software.

3.3 Western Blotting

Western blotting is a widely used technique in cellular and molecular biology to identify the specific proteins from a complex mixture extracted from cells. In western blotting, through a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) a mixture of proteins is separated, according to the molecular weight. After the electrophoresis, proteins are transferred, under an electric field, onto a nitrocellulose or polyvinylidene fluoride (PVDF) membrane and further incubated with an antibody specific for the protein we intend to identify. After the recognition of this antibody with a secondary antibody peroxidase conjugated and the chemo-luminescent development with the peroxidase substrate, luminol; a band for the protein of interest will be revealed.[171]

For western blotting the cells were cultured in 6 well-plate, afterwards cells were collected by scraping or by incubation of PBS (1X) + 2% EDTA. Then cells were centrifuged at 255 × g, cell extracts were performed by lysing cells with Radio-Immunoprecipitation Assay (RIPA) buffer and stored at -20^{0}C.

Cell lysates were centrifuged (Eppendorf) at 10600 × g for 5 min at 4^{0}C and supernatant were collected. Protein concentration was measured through the Bradford method, using Bio-Rad protein assay reagent (500-0006, Bio-Rad) via spectrophometric quantification (595 nm). After quantification of protein, 50 µg of total protein from each sample lysate were loaded to the gel in 5X loading buffer (10% SDS, 0.5% bromophenol blue in Tris-HCL (pH 6.8) and 10% β-mercaptoethanol; M3148, Sigma), after protein denaturation under boiling at 95^{0}C -100^{0}C for 10 min. After that, samples were centrifuged at 10600 × g for 2 mins, placed on ice and then samples were loaded in 12% acrylamide:bis acrylamide (29:1; 161-0156 Bio-Rad) gel (Tris-glycine SDS-

PAGE). Electrophoresis was performed in MINI-PROTEAN Tetra Electrophoresis System (Bio-Rad) at 140 V, into 1X TGS buffer (Tris-Glycine-SDS 10x; 161-0772, Bio-Rad). After that, the gel was transferred to an Immun-Blot® PVDF membrane with a Trans-Blot® TURBO (Bio-Rad), at 25 V, (1,3 A) for 10 min. When membrane transfer is completed, the membrane was blocked with 3% weight (w)/volume(v)) bovine serum albumin (BSA; A9647, Sigma) in PBS (1X) 0,1% volume (v)/volume (v) Tween 20 (PBS (1X) - 0,1% Tween - 3% BSA), for 1 h at room temperature in agitation, to avoid nonspecific binding to membrane. Then the membrane was incubated with primary specific antibody (Rabbit anti-human xCT, ab175186- Abcam; diluted 1:1000 in PBS (1X)- 0.1% Tween - 3% BSA for xCT at 4^0C with agitation, overnight. After the overnight incubation with primary antibody, the membrane was washed 3 times, for 5 min in agitation, with PBS (1X) 0.1% (v/v) Tween 20 to remove unbound primary antibody. Then, secondary antibody was incubated with a primary antibody- species and class specific secondary antibody with IgG-conjugated horse raddish peroxidase (HRP) (anti-rabbit, 31460, from Thermo Scientific or anti-mouse 31430, Thermo Scientific; 1:5000 in PBS (1X)- 0.1% Tween - 3% BSA for 2 h with agitation. After incubation with secondary antibody, the membrane was washed 3 times, for 5 min, with PBS (1X) 0.1% (v/v) Tween 20. To reveal peroxidase activity, ECL method was used and digital images were collected by using ChemiDoc XRS system (Bio – Rad) with image lab software. β-actin was used as endogenous control for protein normalisation, being its levels assessed by incubating the membrane with anti-β-actin (A5441, Sigma Aldrich) 1:5000 in 3% (w/v) BSA in PBS1x, 1% (v/v) Tween 20), overnight at 4^0C. The intensity of each band was measured using Image J software.

3.4 Transfection

Transfection is the process of deliberately introducing naked or purified nucleic acids into eukaryotic cells. Transfection can be mediated by cationic liposomes that will fuse with the cell membrane and delivering the plasmid.

In order to assess the role of xCT in pancreatic cancer chemoresistance QGP-1 and BON-1 cell lines were transfected with *SLC7A11* gene coding sequence, *Homo species* (Genscript) in core plasmid pcDNA3.1/C-(k)DYK , by using the cationic liposome Lipofectamine™ 2000. The Mock variant was transfected with the empty core plasmid pcDNA 3.1^+/ C-(k)DYK. In the same way, the production of *BRAF*, *KRAS* or *NRAS* mutated variants of BON-1 cell line was performed by transfecting cells with plasmid of *BRAF* (PECFP V600E), *KRAS* (PECFP G112V), *NRAS* (PECEP C161R) and Mock (PECEP C3), by using the cationic liposome Lipofectamine™ 2000.

For that purpose, each cell line was cultured in a 25 cm^2 cell culture T-flask, until 80% confluence. For each flask, 1 µg of plasmid of different variant was diluted into 500 µL of serum free Dulbecco's Modified Essential Medium/Nutrient mixture F- 12 (Ham) 1x for BON-1 cell line and Roswell Park Memorial Institute (RPMI) 1640 Medium 1x for QGP-1 cell line. Then 20 µL of Lipofectamine™ 2000 were also diluted into 500 µL of serum free specific medium for T-flask. After that, both suspensions were incubated at room temperature for 5 min and then the two suspensions were mixed and again incubated at room temperature, for 20 minutes, in order to

form plasmid: Lipofectamine™ 2000 complexes. Finally, the complexes (1 mL) were added into each T-flask, replacing the culture medium. After incubation for 4-6 h, at 37^0C, in a humidified 5% CO_2 environment, 2 mL of complete culture medium was added to each T-flask.

After transfection, transfected cells were selected through continuous supplementation of Geneticin (10131027, Invitrogen) to the medium. With an interval of one week, the concentration of selective antibiotic was decreased, order of last concentration that was maintained: 1 mg/mL, 500 µg/mL, 100 µg/mL.

3.5 Immunofluorescence

Immunofluorescence is a widely used technique that depends on the use of antibodies conjugated with fluorescent dye known as fluorophores or fluorochromes (eg fluorescein isothiocyanate- FITC) to detect a specific target antigen, under a fluorescent microscope. In this thesis framework, we performed an indirect method of immunofluorescence where the primary antibody binds to the antigen, followed by the secondary antibody, conjugated with a fluorophore, against the specific isotype of the primary antibody.[172]

To investigate the effect of cysteine, cisplatin and sulfasalazine in xCT expression, cells were cultured in 8 well glass plate. After 16 h of experimental conditions, cells were fixed with 200 µL of 4% of paraformaldehyde (104003, Merck Millipore), incubate for 15 min at RT. Then cells were washed with PBS (1X) and incubated with 50 mM of NH4Cl in PBS (1X) 10 min at RT. To block and permeabilize the cells, PBS 1X – BSA 0.5%- 0.1 % saponin was used during 15 min at RT. After that, cells were incubated with the primary antibody (ab131429-Abcam; Rabbit anti-human xCT) diluted in PBS(1X)- 0,5% BSA -0,1% saponin (1:100) for 30 min. Cells were rinsed with PBS 1X – BSA 0.5%- 0.1 % saponin and incubated with the secondary antibody conjugated with Alexa Fluor® 488 anti-rabbit (A-11034, Invitrogen) during 2 h at RT. To confirm the specificity of secondary antibody, a negative control was used (control without primary antibody) incubated with secondary antibody. Then, cells were washed again with in PBS(1X)- 0,5% BSA -0,1% saponin 3 times. After that, the slides were mounted in VECTASHIELD media containing DAPI (4'-6-diamidino-2-phenylindole) (H1200, Vector Labs). Then, the fluorescence sign was analysed by standard fluorescence microscopy using a Zeiss Imajer.Z1 AX10 microscope.

3.6 Statistical analysis

For all experiments, statistical analyses were performed in GraphPad Prism 5.0 software (www.graphpad.com). Data is presented as the mean ± SD. Assays were performed with, at least, 3 replicates per experimental condition. For comparisons of two groups, two-tailed unpaired T-test was used. For comparison of more than two groups, One-way and Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test were used. Statistical significance was established as $p<0.05$.

4. Results

4.1 Cisplatin induces cell death in BON-1 but not in QGP-1 cell line.

To reveal the effect of cysteine, cisplatin and combination of cysteine and cisplatin in PNETs cell lines, we performed cell death analysis by flow cytometry.

Results have shown that in BON-1 cells, with 16 h of experimental conditions, no differences were found upon cysteine exposure. Moreover, we also observed significant increased cell death levels upon exposure to cisplatin and cisplatin plus cysteine, in comparison to control and cysteine conditions **(figure 4.1 A).**

In QGP-1 cells, with 16 h of experimental conditions, no differences were found upon exposure of cysteine, cisplatin and combination of cisplatin plus cysteine **(figure 4.1B)**

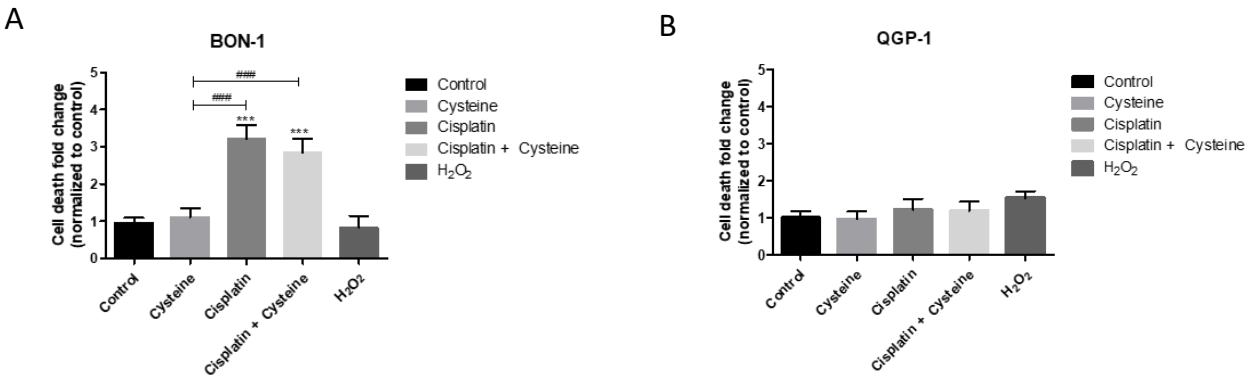

Figure 4.1- The effect of cysteine, cisplatin and cysteine plus cisplatin, in BON-1 and QGP-1 cell lines. After 16 h, cells were collected, and the percentage of cell death was evaluated by flow cytometry. **A**. Cell death in BON-1 cell line- fold change in relation to control. **B.** Cell death in QGP-1 cell line - fold change in relation to control. Results are shown as mean ± SD *p<0.05, **p<0.01, ***p<0.001 (One-Way ANOVA, Tukey). (*) is the statistical significance compared to control, (#) shown the differences between cysteine and cisplatin

4.2 Inhibition of xCT together with cysteine contributes for cisplatin resistance of BON-1 cell line

At first, we confirmed that xCT was expressed in BON-1 and in QTGP-1 **(figure 4.2 A).** To disclose the effect of xCT inhibition, with sulfasalazine or erastin, and cysteine supplementation, in the response to cisplatin, we performed cell death analysis by flow cytometry.

Results have shown that in BON-1 cells, with 16 h of experimental conditions, sulfasalazine itself, but not erastin, significantly induced cell death. Cysteine, erastin or sulfasalazine potentiated cisplatin toxicity, although when cisplatin was combined with cysteine plus erastin or cysteine plus sulfasalazine the basal cell death levels were rescued **(figure 4.2 B).**

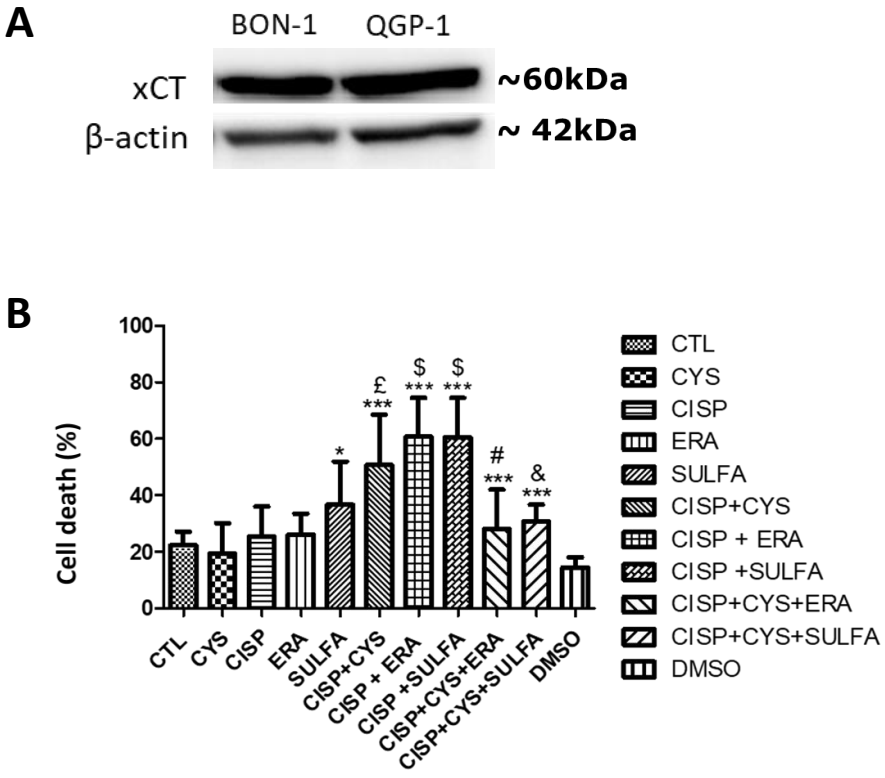

Figure 4.2- The effect of erastin, sulfasalazine and cysteine in the response of BON-1 cell line to cisplatin. A. xCT protein levels, in BON-1 and QGP-1 cell lines. After collection of cells, protein expression levels of xCT were evaluated by western blotting. **B.** After 16 h, cells were collected, and the percentage of cell death was evaluated by flow cytometry. Results are shown as mean ± SD *p<0.05, **p<0.01, ***p<0.001 (one-Way ANOVA, Tukey). (*) indicates the statistical significance compared to control, (£) indicates the statistical significance comparing cysteine and cisplatin, ($) indicates the statistical significance comparing cisplatin with cisplatin plus erastin and cisplatin plus sulfasalazine, (#) indicates the statistical significance comparing cisplatin with cisplatin plus erastin and cisplatin plus erastin plus cysteine, (&) indicates the statistical significance comparing cisplatin plus sulfasalazine and cisplatin plus sulfasalazine plus cysteine.

4.3 Sulfasalazine combined with cysteine and cisplatin stimulates the expression of xCT

To address the effect of cysteine, cisplatin and sulfasalazine, in xCT expression, we performed an immunofluorescence assay.

Through immunofluorescence analysis, with 16 h of experimental conditions in BON-1 cell, results showed a slightly decrease in xCT levels upon exposure of cisplatin **(figure 4.3B)**

All the conditions with sulfasalazine decreased the expression of xCT, except when it was combined with cysteine and cisplatin **(figure 4.4B)**

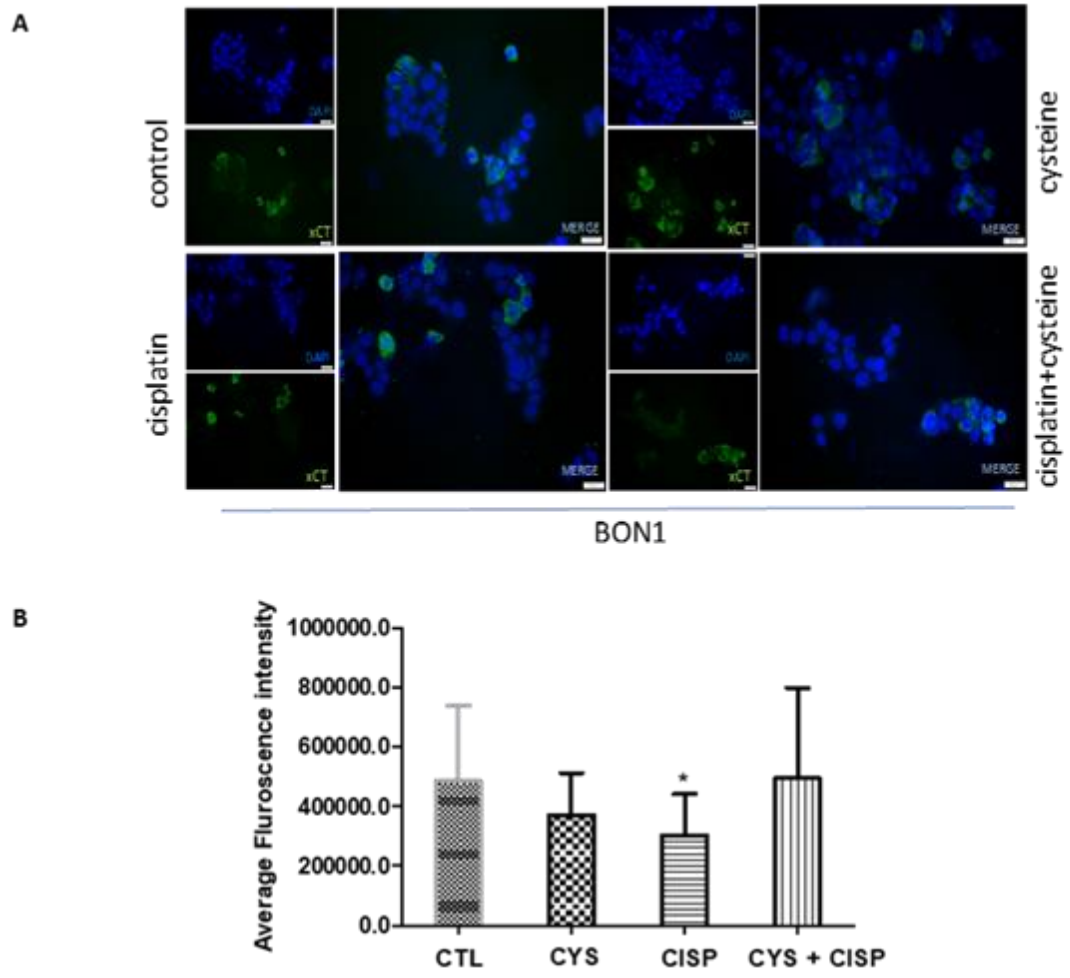

Figure 4.3- Cysteine, cisplatin, cysteine combined with cisplatin do not affect xCT protein levels, in BON-1 cell lines. After 16 h of conditions exposure, the protein level of xCT were analysed. **A.** Fluorescence microscopy (magnification 400 x). Nuclei were stained with DAPI (blue). **B.** Immunofluorescence quantification was done using Image J software in BON-1 cell line. Results are shown in as mean ± SD *p<0.05, **p<0.01, ***p<0.001 . (Two tailed unpaired t-tests).

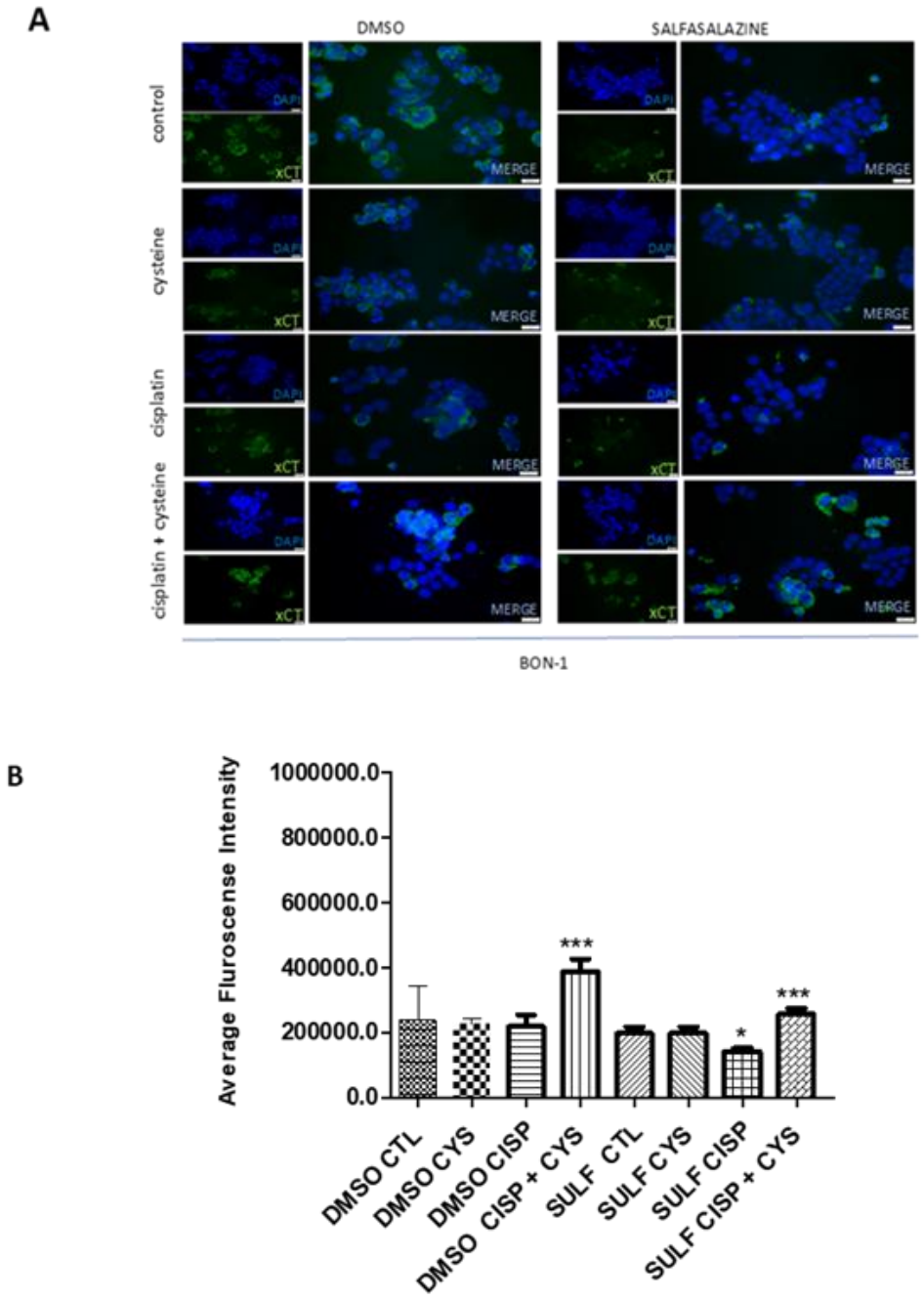

Figure 4.4- The effect of cysteine, cisplatin, cysteine combined with cisplatin and sulfasalazine in xCT protein levels, in BON-1 cell lines. After 16 h of condition exposure, the protein levels of xCT were analysed. **A.** Fluorescence microscopy (magnification 400 x). Nuclei were stained with DAPI (blue). **B.** Immunofluorescence quantification using Image J software in BON-1 cell line. Results are shown in as mean ± SD *p<0.05, **p<0.01, ***p<0.001. (Two tailed unpaired t-tests).

4.4 SeChry, but not SeChry encapsulated and functionalised with folate (SeChry@PURE$_{G4}$-FA), induces cell death in PNETs cell lines

In order to determine the effect of seleno-Chrysin (SeChry), in BON-1 cell line, cells were exposed to free SeChry and SeChry encapsulated and functionalised with folate (SeChry@PURE$_{G4}$-FA). A cell death quantification was performed by flow cytometry.

Result have shown that in BON-1, increased cell death levels were observed upon exposure of SeChry, whereas we did not observe any significant differences with SeChry@PURE$_{G4}$-FA exposure in relation to control **(figure 4.5)**

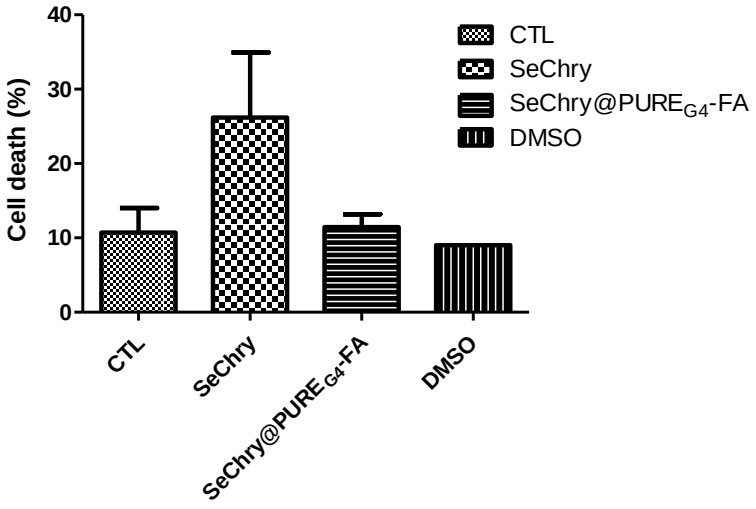

Figure 4.5 The effect of SeChry and SeChry@PURE$_{G4}$-FA in BON-1 cell line. After 24 h of exposure, cells were collected and the percentage of cell death in BON-1 cell line was evaluated by flow cytometry.

4.5 Buthionine sulfoximine encapsulated and functionalised with folate (BSO@PURE$_{G4}$-FA) induces cell death in PNETs cell lines

In order to assess the effect of BSO in PNETs in the reversion of cisplatin resistance, cells were exposed to BSO@PURE$_{G4}$-FA alone or combined with cysteine and/or cisplatin. Cell death was measured by flow cytometry.

Results have shown that BSO@PURE$_{G4}$-FA induces cell death in BON-1 and QGP-1 cells, independently of cysteine and cisplatin **(figure 4.6).**

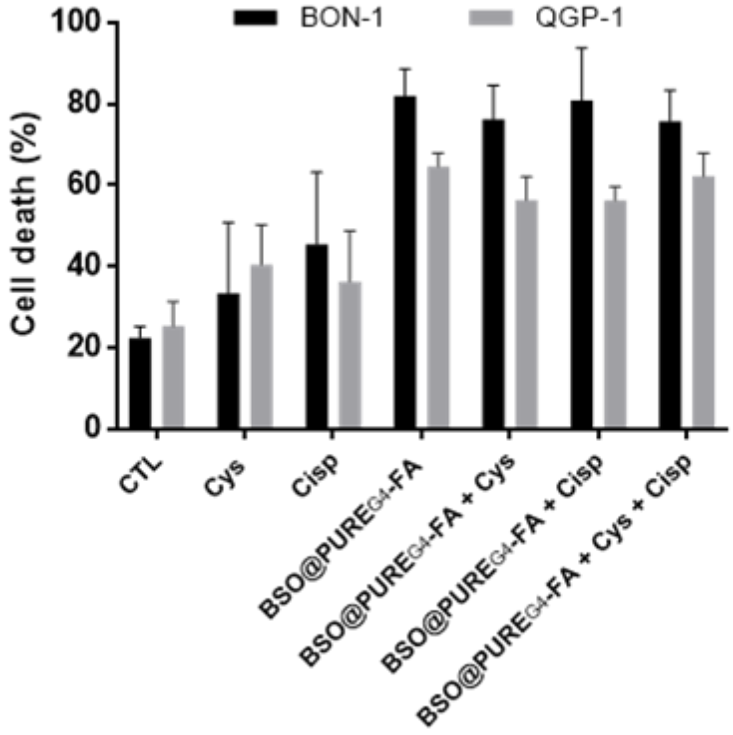

Figure 4.6 The effect of BSO@PURE$_{G4}$-FA combined with cysteine and cisplatin in PNETs cells (BON-1 & QGP-1 cell lines). After 16h of experimental conditions, cells were collected to perform the cell death analysis by flow cytometry.

4.6 Failure in the transfection of BON-1 cell line with *NRAS*, *KRAS* and *BRAF* mutated variants (*BRAF*- V600E, *KRAS*-G112V, *NRAS*-C161R)

We observed no alteration in the levels of phosphor-Erk (pErk), meaning that MAPK pathways was not altered in BON-1 cells transfected with *NRAS*, *KRAS* and *BRAF* mutated variants. A possible explanation is the fact that this cell line is already mutated for *NRAS*- Q61R, being the increased stimulation of MAPK pathway a phenomenon incompatible with cell survival which impeded the selection of clones overexpressing the mutated variants.

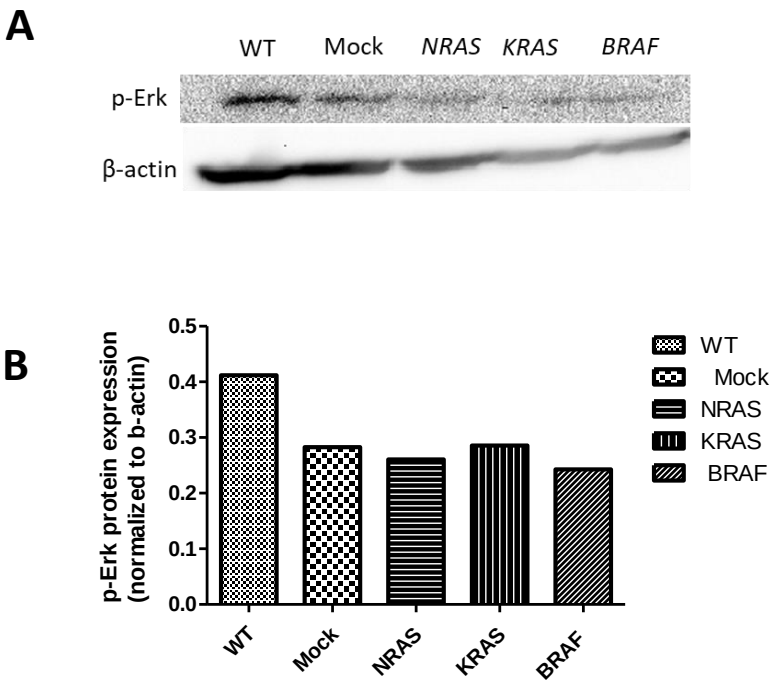

Figure 4.7 p-Erk levels indicate that MAPK pathway has a lower, but non- significant, flow in transfected cells. After collection of BON-1 cells selected with Geneticin (G418), protein expression level of p-Erk was evaluated. A. Western Blot membrane, β-actin was used for normalization. B. western blot quantification through Image j.

4.7 Failure in the overexpression of xCT.

Due to technical problems, we were not able to get positive transfected cell clones overexpressing xCT. In ana attempt to solve the problem, assays co-overexpressing xCT and its partner 4F2hc (*SLC3A2*) will be performed in the future, since the xCT stabilization and insertion in the cell membrane depends on the interaction with 4F2hc. [173]

5. Discussion

Pancreatic neuroendocrine tumour (PNETs) is one of the rare tumours with a rising incidence day by day. Metabolic reprogramming nowadays has been recognized as a hallmark of cancer which causes a molecular remodelling not only in cancer cells but also in non-cancerous cells, since they share the same microenvironment.[174] The major significance of metabolic reprogramming is to supply energy for proliferation and maintain the redox balance in tumour cells, protecting them against the oxidative stress. Collecting studies present that metabolic reprogramming is being linked to mechanisms of drug resistance in cancer and a rapid development of new strategies of anticancer drug is needed to overcome the chemoresistance.[175]

Chemoresistance is a complex framework with multiple mechanisms which is influenced by the microenvironment as well as by the biology of the tumour.[176] In this context, GSH acts as a contributing factor by interacting with chemotherapeutic drugs and its depletion is exhibited as an effective approach in the sensitization of various types of cancer.[177,178,179] GSH directly targets ROS, being the most important ROS scavenging system. Cysteine is fundamental as a rate limiting substrate and the thiolic component of GSH.[180,181,182]

Cisplatin induces cell death in BON-1 but not in QGP-1 cell line.

The effectiveness of drugs is based not just on their capacity to induce DNA damage but also on the cells capacity to distinguish and respond to DNA damage. Following DNA damage, cells possibly will either repair the damage and re-enter the cell cycle or on the off chance the cells cannot fix the damage and proceed to die.[183]

The platinum-based compound cisplatin is a chemotherapeutic compound generally utilized to treat cancer, which acts principally by means of DNA damage, directly through the formation of adducts or indirectly through the generation of ROS, and ensuing induction of apoptotic programs.[180,184] The antineoplastic pathway of cisplatin possibly will activate several equivalent pathways, leading to apoptosis and cell cycle arrest, based on treatment conditions, types of cells or concentration.[177]

To access the influence of cysteine in cisplatin induced cell death in PNETs cell lines, we exposed cancer cells to these compounds in separate and in combination. In extended exposure time (16 h), cisplatin induced significant cell death in BON-1 cell line but not in QGP-1 cell line **(figure 4.1).** The presence of cysteine did not interfere with the effect of cisplatin.

Our team already found that cysteine was able to inhibit the effect of platinum salts in ovarian cancer, but in PNETs cell lines we did not see cysteine as a cell protector or as capable of overcoming platinum drugs effect. Recent studies showed that members of the oxaliplatin family, such as cisplatin and carboplatin, exhibit cytotoxicity in a broader spectrum in different cancer treatment such as colon, pancreas, breast, prostate and melanoma.[185,186,187] The platinum-containing drug cisplatin combined with capecitabine in PNETs response rates are approximately

30%. [188,189] In melanoma, cisplatin is the most commonly used. [190] Cisplatin-DNA adducts lead to a conformational change of the DNA, permitting protein binding of molecules including high mobility group domains, which ultimately impairs DNA replication and transcription.[191,192] As mentioned, cisplatin induces generation of ROS, that consequently leads to decreased GSH and ultimately causes cell death.[180,184] Our results suggest that BON-1 cells may not rely efficiently on thiols to overcome cisplatin effects at least on the culture conditions and cysteine concentration we tested.

xCT plays a role in cisplatin resistance of BON-1 cell line

GSH molecule consist of cysteine, glutamic acid and glycine residues which plays an important role for the maintenance of intercellular redox balance and detoxification. Consequently, GSH levels show a positive correlation with chemoresistance.[193] Cysteine is principally obtained from extracellular environment by system xc−, having a role as a raw material of intercellular GSH synthesis, closely related to the expression and function of xCT.[105,194] Recent studies have suggested that xCT expression is associated with the development of pre-neoplastic lesions, cancer, poor prognosis and chemoresistance.[195,106] The greater portion of the extracellular cysteine is oxidized to cystine which is responsible for up taking cysteine positively.[196] Recent studies exhibit evidence for the presence of specific transporters for cysteine.[197] In addition, extracellular breakdown of glutathione produces glutamate, glycine, and cysteine discretely, which are again transported into the cell. Accumulating evidence recommended the existence of transporters capable of cysteine-specific uptake.[198,199] The role of xCT, a cystine transport system, causes increased levesl of GSH, involved in the process of chemoresistance. But the inhibition of xCT function also interferes with its expression. Sulfasalazine and erastin are well known inhibitors of this transport system.[108,109,110]

To reveal the modulation of xCT expression, with cysteine, cisplatin together with sulfasalazine and erastin, which contribute to cisplatin resistance in BON-1 cell lines, we performed cell death assay. For xCT transporter, by flow cytometry analysis, we observed in BON-1 cell lines that sulfasalazine itself decreases the expression of xCT (**figure 4.4 B**). As shown in the present paper, xCT expression affects the progression of tumour and Sulfasalazine, inhibitor of xCT, increased cellular oxidative stress,[200] that ultimately reduces the GSH concentration (**figure 4.4 B**). This can be explained by the interconnection between the regulatory mechanisms controlling the expression of xCT and other cystine/cysteine transporters, such as EAAT3, which expression/function regulates the mTOR activity that will control ATF4 action on xCT expression. [201] Since, EAAT3 is itself regulated by mTOR,[202] a feedback positive mechanism can be activated upon xCT inhibition, in which a loop of interactivation between mTOR and EAAT3 expression is exacerbated **(FIGURE 5).**

Interestingly, cisplatin induced a significant decrease in xCT expression (**figure 4.3 B**), which makes a link between the putative ferroptotic cell death induced by cisplatin, since xCT is considered an inhibitor of ferroptosis [118] and the use of inhibitors, such as erastin, stimulates

ferroptosis.[203,118] This is supported by our results showing that cells exposed to cisplatin plus sulfasalazine or erastin present higher levels of cell death (**figure 4.2 B**), as it was already observed in other cancer contexts.[204] Furthermore, when cisplatin was combined with cysteine plus erastin or cysteine plus sulfasalazine a decrease in cell death was shown (**figure 4.2 B**). This phenomenon could be explained by substantial amounts of cysteine to be handled by cancer cells, in order to be taken up to produce enough GSH and abrogate cisplatin toxicity.[197,205] According to this, cysteine was able to rescue xCT expression, in cells exposed to the combination of sulfasalazine, cisplatin and cysteine (**figure 4.4**). As previously mentioned, xCT expression is induced by oxidative stress effectors, as hydrogen peroxide and sodium arsenite, ultimately leads to increased GSH production.[206] Therefore, upon drugs induced oxidative stress, cysteine is needed as a precursor of GSH, that is why the mechanisms regulating the expression of Xct, as mentioned before, involving EAAT3, a cysteine specific transporter, can act on the direction of increasing cysteine uptake.[207]

It is known that cysteine is necessary for the maintenance of cellular homeostasis and survival of cancer cells. [208,209,181] Cysteine, besides being a precursor of GSH, is used for the production of amino acid taurine, having a role in mitochondrial function and also in the control of cellular osmolarity, hydrogen sulphide (H_2S) production, iron-sulfur clusters formation in cell respiration and also acts as a co enzyme.[210,211] Cysteine uptake can be done by xCT transporter, metabolized in the mitochondria via CBS, CSE (dependent on its translocation from the cytosol that is described under stress conditions) and MpST conjugate with CAT activity (already located in the mitochondria), associated with H_2S and ATP production.[212,213]

In such manner, our results suggested that system xc− antiporter (sulfasalazine) can reduce mitochondrial dysfunction-enhanced cisplatin resistance, signifying that higher intracellular GSH levels caused by the system xc− antiporter might contribute to chemoresistance.[214]

Our results pave new research paths in order to know in depth the role of cysteine bioavailability, mediated by its transporters (eg xCT), in platinum salts- induced cell death and in the definition of new therapeutic strategies **(figure 5)**. However, the actual occurrence of ferroptosis in our model should be further investigated.

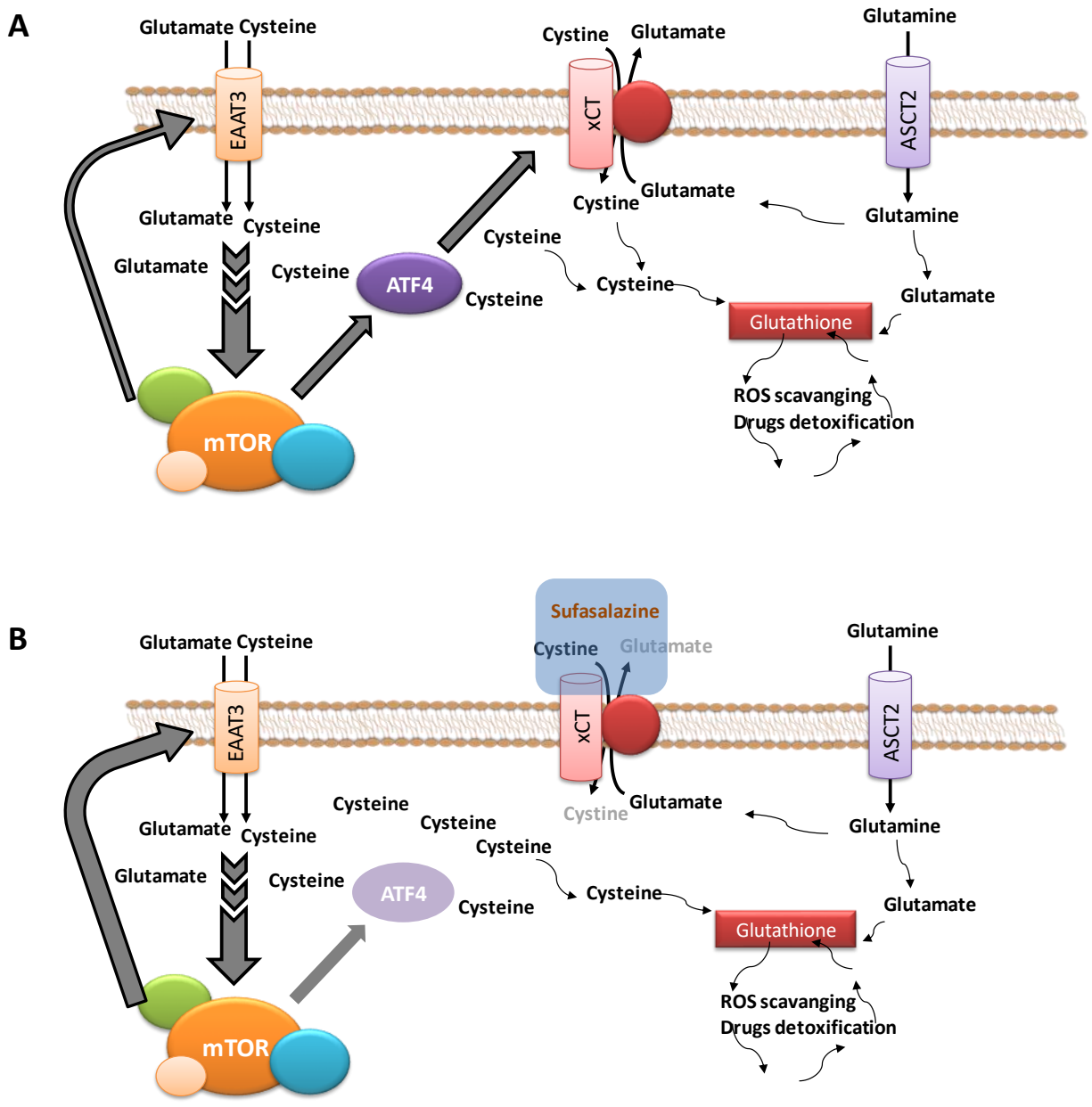

FIGURE 5. Cooperation between different cystine/cysteine transporters ensures chemoresistance. A. The interconnection between the regulatory mechanisms controlling the expression of xCT and other cystine/cysteine transporters, such as EAAT3, which function regulates the mTOR activity and xCT expression, through the action of ATF4.[201] EAAT3 is itself regulated by mTOR.[202] **B.** Upon xCT inhibition, a feedback loop is activated switch on interstimulation of mTOR and EAAT3, enhancing EAAT3 expression and cysteine uptake to sustain GSH synthesis and chemoresistance**.**

SeChry, but not SeChry@PURE$_{G4}$-FA, induces cell death in PNETs cell lines

Selenium (Se) compounds in cancer research over last four decades have received attention as anticancer agents due to their pro-oxidant properties at higher levels.[215,216] Normally, inorganic Se compounds exert a lower cancer therapeutic window, inducing higher systemic toxicity and higher metastatic burden. On the other hand, organic selenium compounds are a vast group of chemically diverse nucleophilic molecules, having significant anti-tumour activity accompanied by higher ability to prevent metastasis and lower systemic toxicity.[217,218] SeChry is an organoselenium compound, which has become a promising candidate in cancer therapeutics, exerting an anti-tumoral effect.[215] It is known that by inducing oxidative stress in cancer cells via redox modulation provides a new therapeutic window for anticancer therapy.[219] Organoselenium compounds act as a redox modulators, exhibiting a higher selectivity and sensitivity in cancer cells;[215,216,220] and they have been considered as xCT inhibitors.[127,87] Based on the antitumoral effect of selenium- chrysin (SeChry), our third aim focused on the effect of new nanoformulations in order to disturb cysteine uptake by using Sechry. To establish our objectives, we conducted a cell death assay by using free SeChry and targeted delivery SeChry using nanoparticles functionalised with folate, SeChry@PURE$_{G4}$-FA, in BON-1 cell line. Results have shown that in BON-1, increased cell death levels were observed upon exposure to SeChry but not to SeChry@PURE$_{G4}$-FA (**figure 4.5**). This cell death conducted by SeChry could be explained by the newly recognized pro-oxidant role of selenated-compounds, which is based on ROS generation, oxidation of protein, thiols and direct or indirect DNA binding, ultimately leading to apoptotic cell death.[215]

The lack of cell death with SeChry@PURE$_{G4}$-FA exposure can be related to the low expression of the folate receptor. The evaluation of folate receptor expression must be addressed in our cancer cell models. However, some studies have pointed the neuroendocrine tumours as suitable for the therapies using folate functionalised particles for targeted therapy.[221]

Buthionine sulfoximine encapsulated and functionalised with folate (BSO@PURE$_{G4}$-FA) induces cell death in PNETs cells

Buthionine sulfoximine (BSO) a synthetic amino acid, irreversibly inhibits the γ-glutamyl-cysteine synthetase, which catalyses the rate limiting step of GSH biosynthesis.[222] Increased GSH also has an association with reduced drugs sensitivity of tumour cells due to acquired or intrinsic resistance of some anticancer drugs, specially platinum salts.[223,224]By depleting the GSH synthesis, since it plays an important role in protecting cells against oxidative stress, ultimately it will result in free- radical induced apoptosis.[225]

To reveal the effect of disturbing GSH synthesis (BSO@PURE$_{G4}$-FA) in the response to cisplatin, we conducted a cell death assay in PNETs cell lines. In BON-1 and QGP-1 cells, significant increased cell death levels were induced by in BSO@PURE$_{G4}$-FA compared to control **(figure 4.6).** The combination of BSO@PURE$_{G4}$-FA, cisplatin and cysteine also induced a significant increase in

cell death in relation to cisplatin and cysteine alone. Cysteine was not able to reduce BSO@PURE$_{G4}$-FA and cisplatin toxicity **(figure 4.6),** suggesting that BSO was able to inhibit GSH synthesis having no room for cysteine intervention in order to enhance the levels of GSH and consequently the resistance to cisplatin. Similar results have been observed by our team in an ovarian cancer model.[226]

The cell death conducted by BSO@PURE$_{G4}$-FA in PNETs cell lines could be explained by delivering BSO more efficiently to cancer cells allowing the decrease in the concentration of BSO applied. Platinum salts such as cisplatin respond quickly with nucleophilic sulfur-containing molecules, in this way a higher concentration of GSH will be able to capture cisplatin. By diminishing the pool of GSH, cisplatin can bind to DNA, which ultimately causes cell death.[227]

6. Conclusions

The limitation of the success of systemic anticancer therapy has been chemoresistance, decreasing overall survival. This problem is obvious after using chemotherapy for more than half a century and keeps on being a considerable issue in the present time of molecularly targeted drugs and personalized medicine. So, new targeted therapy can be generated to disclose what are the phenomena beyond the mechanism of chemoresistance.

Pancreatic Neuroendocrine tumours (PNETs) are a rare heterogenous group of slow growing tumours. The chemoresistance behaviour of these tumours depends on a set of multiple mechanisms, specially the availability of chemotherapy agents in which ROS-scavenging systems act via increasing levels of GSH. To understand the cysteine driven molecular pathway in cancer cell resistance, research works have been investigating the role of cysteine transporters, so that they can be able to design new therapeutic strategies to fight cancer progression. The main objective of our work allowed to reveal the role of xCT transporter and the role of cysteine in PNETs cell line resistance. This cell line showing different response patterns in cysteine transporters activity helped to reveal the differences of the transporter in chemoresistance mechanism. It also showed that beside xCT transporter other cysteine transporter such as EAAT3 also appeared to be involved in the dynamics of chemoresistance mechanism.

This work was also important to undercover the effect of new nanoformulations in order to disturb cysteine uptake by using SeChry and to inhibit GSH synthesis by using BSO in PNETs cell lines. SeChry, but not SeChry@PURE$_{G4}$-FA, induced cell death in BON-1 cell lines. SeChry cytotoxicity can be selective for cancer cells and this was taken in consideration in our new strategy by using SeChry@PURE$_{G4}$-FA, however the assay was not successful and new markers for targeted delivery must be investigated in PNETs.

BSO@PURE$_{G4}$-FA induced cell death in combination with platinum salts in PNETs cell lines. Possibly, the use of folate functionalised particles will help to bypass the critical step in the non-specific delivery of BSO to non-cancer cell. The targeted BSO delivery to cancer cells can be explored as a novel strategy in cancer therapeutics.

Moreover, more assays with cancer and non-cancer cells must be done in order to determine if folate receptor is in fact a suitable target to delivery drugs to PNETS cells, and find new and more specific targets.

7. Future perspectives

Our study paved the path for some new cancer reaserch lines in the context of cancer metabolism and chemoresistance in PNETs. So as future perspectives, it would be interesting:

- To study in depth the effect of xCT transporter, upon SeChry and cisplatin exposure.
- To evaluate the EAAT3 transporters modulation, upon exposure of sulfasalazine and erastin, xCT inhibitors.
- To evaluated the metabolic modulation and response to therapy in cells co-overexpressing xCT and 4F2hc.
- To reveal the cytotoxic effect of SeChry in non-malignant cell lines.
- To verify the effect of xCT and EAAT3 transporter modulation upon exposure of cysteine and cisplatin.
- To reveal the expression of enzymes involved in cysteine metabolism and glutathione synthesis in PNETs sections by immunohistochemistry.
- To address the cysteine metabolism in different KRAS/BRAF genetic backgrounds, commonly altered in PNETs.

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APPENDICES

Solutions used in the experimental work

PBS 10X (pH 7.4-7.6)

1 L= 80 g NaCl (1,37 M) (106404, Merck)

2 g KH2PO4 (14,7 mM) (104873, Merck)

11,1 g Na2HPO4 (78,1 mM) (S-0876, Sigma)

2 g KCl (26,8 mM) (104936, Merck)

1L= ddH2O

PBS(1X) – 0,1% (v/v) Tween 20

1 L= 1X PBS 1 L

1 mL Tween 20 (20605, USB)

PBS(1X) - 0,1% (v/v) Tween 20 - 3% (w/v) BSA

PBS 1X – 0,1% (v/v) Tween 100 mL

3 g BSA (A9647, Sigma)

PBS(1X) – 0,5% (w/v) BSA – 0,1% (w/v) saponin

For100 mL

100 Ml PBS1X

0,5 g BSA (A9647, Sigma)

0,1 g saponin

Propidium Iodide (PI) solution (50 µg/mL)

For 50 mL:

1 mL of 2.5 mg/mL PI solution (P4170, Sigma) (prepared in 1X PBS)

49 mL 1X PBS

Annexin binding buffer

1X 0.01 M Hepes (pH 7.4) (391333, Millipore)

0.14 M NaCl (106404, Merck)

2.5 mM CaCl2 (449709, Sigma)

Transfer buffer

For 500mL:

7,5 g glycine (US16407, USB)

1,5 g Trizma-base (T-8524, Sigma)

100 mL Metanol (107018, Merck)

0,5mL 10% SDS (V6551, Promega)

ddH2O to 400 mL

5X SDS gel loading buffer

250 mM Tris HCl (pH 6,8) (0,5M 161-0799, Bio-Rad)

10% SDS (V6551, Promega)

0,5% bromophenol blue

50% glycerol (1.04094.1000, Merck)

PBS(1X) – 0,1% (w/v) BSA

For 100 Ml

PBS 1X 100 mL

0,1 g BSA (A9647, Sigma)

RIPA buffer

For 10 mL

20 mM Tris-HCl pH 7,5

150 mM NaCl (106404, Merck)

5 mM KCl (104936, Merck)

5 mM MgCl2 (M-8266, Sigma)

1% Triton X-100 (T8787, Sigma)

1 Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablet (11836170001, Roche)

1 mM Orthovanadate (Na3VO4)

1 mM Sodium fluoride (NaF) (201154, Sigma)

ddH2O to 10 mL